Original Article

Effects of Interferon-γ on odontoblastic differentiation and mineralization of odontoblast-like cells

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Introduction: Dentinogenesis is regulated by cytokines and growth factors, and modulated by alterations in the extracellular microenvironment. Dental matrix protein-1 (DMP-1), which is predominantly expressed in odontoblasts, is required during the early and late stages of odontogenesis. In the present study, we examined the involvement of proinflammatory cytokines in the expression of odontogenic markers in the rat odontoblast-like cell line KN-3.

Methods: The expression of DMP-1 and p38 mitogen-activated protein kinase (MAPK) in proinflammatory cytokine-treated KN-3 cells was evaluated by immunoblot analysis. Alkaline phosphatase (ALP) activity in proinflammatory cytokine-treated KN-3 cells was measured by ALP staining and a colorimetric assay.

Results: DMP-1 protein was downregulated in KN-3 cells treated with interferon-γ (IFN-γ) for 3 days, but not in interleukin-1β (IL-1β)-treated cells. The IFN-γ-induced downregulation of DMP-1 was rescued by treatment with IL-1β for 3 days. Interestingly, ALP activity was also suppressed in IFN-γ-treated KN-3 cells and no significant change was induced by IL-1β treatment for 5 days. In addition, IFN-γ treatment for 3 days remarkably upregulated the phosphorylation of p38 MAPK in KN-3 cells.

Conclusions: INF-γ treatment downregulated DMP-1 expression and ALP activity in KN-3 cells through prolonged phosphorylated p38 MAPK. IL-1β treatment restored these odontogenic reactions mediated by IFN-γ. Taken together, these findings suggest that IFN-γ and IL-β may be involved in the complex regulation of odontogenesis in the microenvironment of dental pulp.

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Introduction

Dental pulp is a vascular connective tissue enclosed in rigid mineralized dentin. Dental pulpitis is a very common inflammatory disease caused by carious bacteria or trauma, resulting in an inflammatory response characterized by accumulation of macrophages, lymphocytes and leukocytes.

These immune cells release proinflammatory cytokines and inflammatory mediators such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-6, and prostaglandins to induce acute and chronic inflammation in the dental pulp tissue[1].

IFN-γ is an important proinflammatory cytokine that is mainly released from T cells in response to antigen stimulation, which activates macrophages. An unmethylated IFN-γ gene has been observed in inflamed dental pulp tissues by epigenetic analysis[2]. Moreover, IFN-γ is involved in the initial pulpal response to dental caries[3]. It is also known that the early inflammatory reaction has a protective effect on dental pulp tissue viability through expression of osteonectin (ON), osteocalcin (OC), bone sialoprotein (BSP), and dental matrix protein-1 (DMP-1) in osteoblastic cells stimulated with IL-1β and TNF-α[4-6]. On the other hand, long-term treatment with IL-1β and TNF-α inhibits cell mineralization through downregulation of ON, OC, BSP, and DMP-1 expression in dental pulp cells[6, 7].

DMP-1 was initially identified as one of the acidic phosphoproteins in odontoblasts during embryonic and postnatal development[8]. It is expressed predominantly in odontoblasts of teeth and osteocytes in bone[9-11]. Using DMP-1-knockout mice, previous studies have reported that DMP-1 regulates the mineralization of hard tissues such as bone and dentin, indicating that DMP-1 expression is required in both the early and late stages of odontogenesis[12, 13]. Overexpression of DMP-1 induces differentiation of embryonic mesenchymal cells into odontoblast-like cells, resulting in accelerated mineralization[14]. These findings suggest that DMP-1 is an important regulatory mediator of odontoblastic differentiation as well as mineralization of dentin.

Previously, we established a proliferating dental pulp progenitor cell line, KN-3, from dental papilla cells of rat incisors[15]. We reported that KN-3 cells express high levels of alkaline phosphatase (ALP) activity, as well as runt-related transcription factor 2 and dentin sialophosphoprotein[15-17]. In addition, we showed that KN-3 cells form mineralized nodules by treatment with ascorbic acid and β-glycerophosphate in an *in vitro* culture system. Because KN-3 cells exhibit typical odontoblastic properties, we analyzed the precise molecular mechanisms by which KN-3 cells differentiate into mature odontoblasts[15, 18, 19].

In the present study, we found downregulation of DMP-1 expression and ALP activity in IFN-γ-treated KN-3 cells, and that IL-1β treatment rescued the suppressive effects induced by IFN-γ. Interestingly, phosphorylation of p38 mitogen-activated protein kinase (MAPK) was prolonged in IFN-γ-treated KN-3 cells for 3 days. These findings suggest that downregulation of DMP-1 expression and ALP activity induced by IFN-γ treatment is due to the prolonged phosphorylation of p38 MAPK in KN-3 cells.

Materials and Methods

1)Cell culture

KN-3 cells were cultured in α-minimum essential medium (Gibco, Gland Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (JRH Bioscience, Lenexa, KS), 100 μg/ml streptomycin (Sigma-Aldrich, St Louis, MO), and 100 U/ml penicillin G (Sigma-Aldrich) at 37°C with 5% CO_2[15].

2)Reagents and antibodies

Recombinant human IFN-γ and recombinant human IL-1β were obtained from R&D systems (Minneapolis, MN). A polyclonal antibody against DMP-1 was purchased from GeneTex Inc. (Irvine, CA). A monoclonal antibody against β-actin was purchased from Sigma-Aldrich. Polyclonal anti-phospho-p38 MAPK and anti-p38 MAPK antibodies, and monoclonal anti-c-jun, anti-phospho-c-jun, anti-p44/42 MAPK (Erk1/2), and anti-phospho-p44/42 MAPK antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). As secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from GE Healthcare (Little Chalfont, UK).

3)Cell viability assay

Cell viability was determined using a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan). KN-3 cells (1×10^4 cells/well) were seeded in 96-well plates and cultured for 6 h. The cells were then stimulated with IFN-γ and IL-1β for 48 h. WST-8 solution (10 μl) was the added to each well, followed by incubation for 2 h. Absorbances at 450 nm and 630 nm were measured using a Multiskan JX Microplate Reader (Thermo Scientific, Rockford, IL).

4)Immunoblot analysis

KN-3 cells (1×10^5 cells/ml) were seeded in 100-mm dishes and treated with INF-γ (25 ng/ml) and IL-1β (25 ng/ml) for the indicated times. Following incubation, total protein was extracted using sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-HCl containing 2% SDS; pH 6.8). Protein concentrations were measured using a DC protein

assay kit (Bio-Rad, Hercules, CA). Protein samples (20 μg) were subjected to electrophoresis on SDS-polyacrylamide gels and then electroblotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Nonspecific binding sites were blocked by immersing the membranes in Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature. The membranes were incubated with diluted primary antibodies at 4°C overnight and then HRP-conjugated secondary antibodies for 1 h at room temperature. After washing the membranes, immunodetection were performed using ECL western blotting detection reagent (Amersham Pharmacia Biotech, Uppsala, Sweden) and captured digitally with a Gel DocTM XR Plus System (Bio-Rad, Hercules, CA). Densitometric analysis of protein bands in western blots was performed with Image Lab® (Bio-Rad, Munich, Germany).

5)ALP assay

KN-3 cells (2×10^3 cells/well) were seeded in 6-well plates and treated with IFN-γ and IL-1β for 5 days. The adherent cells were then fixed and stained for ALP using a TRAP/ALP stain kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) according to the manufacturer's instructions. At each time point after IFN-γ and IL-1β stimulation, ALP activity was measured using a *p*-nitrophenylphosphate assay (LabAssay ALP Kit; Wako Pure Chemical Industries, Ltd). After 15 min of incubation at 37°C, absorbances at 405 nm were determined using the Multiskan JX microplate reader to calculate the specific activities of ALP (U/μl). One unit of enzymatic activity is defined as the release of 1 nmol *p*-nitrophenyl per minute at pH 9.8 and 37°C. All images were captured using pre-determined light intensity at the same magnification. Image Lab® software (Bio-Rad, Munich, Germany) was used to generate percent stained values for each field of view.

6)Statistical analysis

All data expressed as the mean ± standard deviation of three experiments, with similar results obtained in each experiment. Statistical differences were determined using the unpaired Student's *t*-test. $p<0.05$ was considered statistically significant.

Results

1)Expression of DMP-1 in KN-3 cells

No change in the expression levels of DMP-1 protein

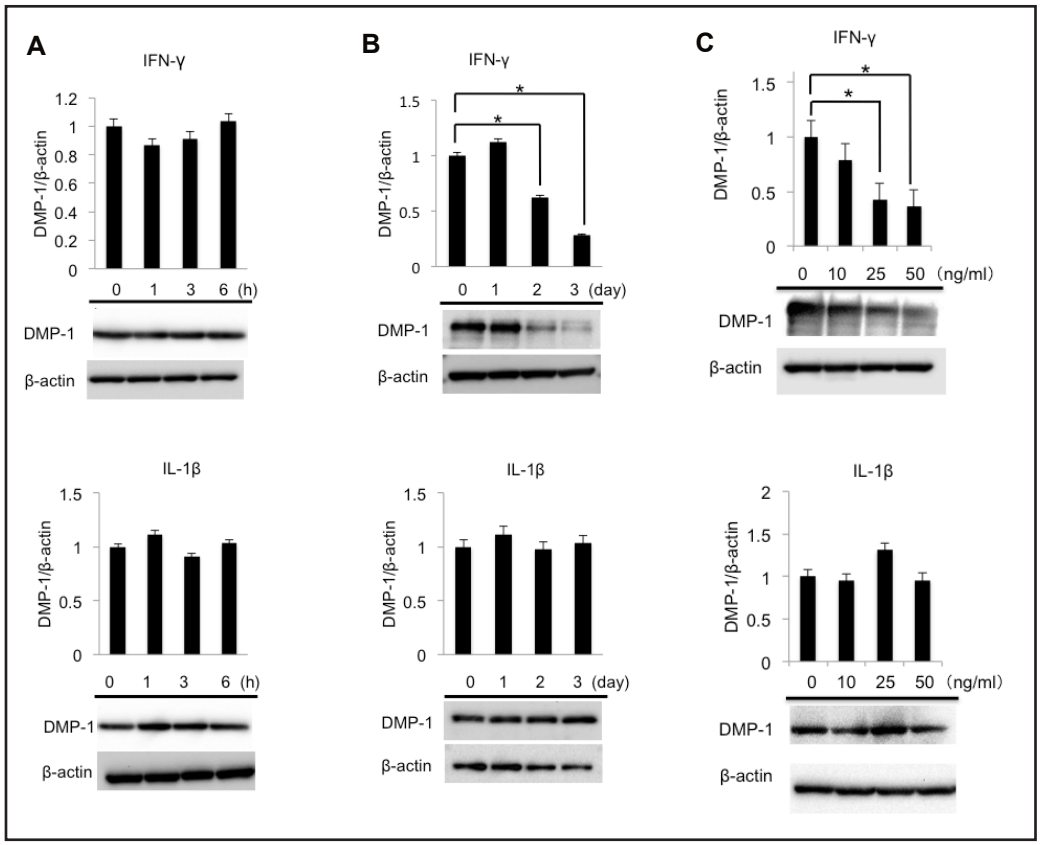

Fig. 1 Expression of DMP-1 in KN-3 cells

(A)Detection of DMP-1 expression in KN-3 cells treated with IFN-γ (25 ng/ml) and IL-1β (25 ng/ml) at the indicated time points by immunoblot analysis (short-term culture). (B)Detection of DMP-1 expression in KN-3 cells treated with IFN-γ (25 ng/ml) and IL-1β (25 ng/ml) at the indicated time points (long-term culture). (C)Detection of DMP-1 expression in KN-3 cells treated with IFN-γ and IL-1β at the indicated concentrations for 2 days by immunoblot analysis.

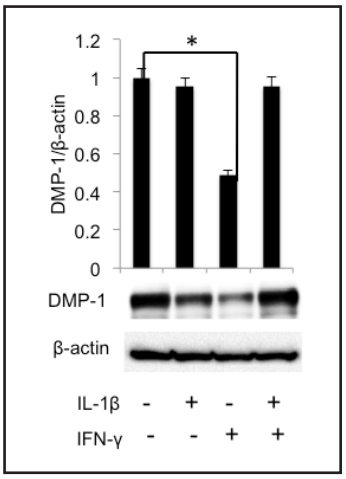

Fig. 2 Effects of IFN-γ and IL-1β on DMP-1 protein expression

KN-3 cells were stimulated with INF-γ (25 ng/ml), IL-1β (25 ng/ml), or both INF-γ (25 ng/ml)/IL-1β (25 ng/ml) for 3 days. The protein expression of DMP-1 was determined by immunoblot analysis.

was detected in KN-3 cells treated with IFN-γ or IL-1β for 6 h (Fig. 1A). However, IFN-γ (25 ng/ml) treatment for 2 or 3 days significantly decreased the expression of DMP-1 in KN-3 cells. In contrast, DMP-1 expression was detected continuously at stable levels in IL-1β (25 ng/ml)-treated KN-3 cells (Fig. 1B). Western blotting analysis were performed at 0, 10, 25, 50, 100 ng/ml for 2 days in IFN-γ- and IL-1β-treated KN-3 cells. The expression of DMP-1 in INF-γ-treated KN-3 cells were downregulated in a dose-dependent manner (Fig. 1C). Pretreatment of IFN-γ and treatment of IL-1β for one or two days later in KN-3 cells showed no significant difference on the expression of DMP-1 (data not shown). WST-8 assay results showed that IFN-γ and IL-1β treatments of up to 100 ng/ml for 3 days had no effect on KN-3 cell viability (data not shown).

2)Cooperative effects of IFN-γ and IL-1β on DMP-1 protein expression in KN-3 cells

To explore how INF-γ (25 ng/ml) and IL-1β (25 ng/ml) is involved in the expression of DMP-1 in KN-3 cells, we examined the cooperative effect of these proinflammatory cytokines on DMP-1 expression in KN-3 cells. The protein expression of DMP-1 was downregulated in KN-3 cells treated with IFN-γ for 3 days, but not in IL-1β-treated KN-3 cells. Interestingly, IL-1β treatment restored the expression level of DMP-1 up to the stable expression level of untreated cells (Fig. 2).

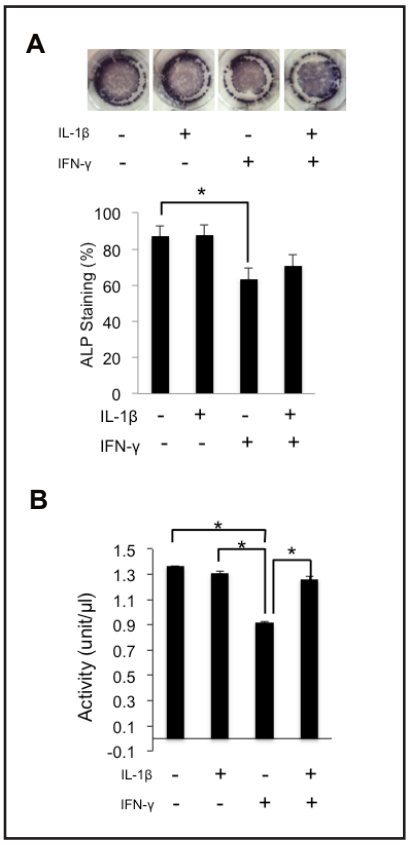

Fig. 3 Effect of IFN-γ and IL-1β on ALP activity in KN-3 cells

KN-3 cells were treated with INF-γ (25 ng/ml), IL-1β (25 ng/ml), or both INF-γ (25 ng/ml)/IL-1β (25 ng/ml) for 5 days.

(A)Cells were fixed and stained for ALP as described in the Materials and Methods. 10 fields of view per sample were captured and percentage area of staining was calculated. Data represent means ± SE of three independent experiments.

(B)KN-3 cells were treated with IFN-γ (25 ng/ml), IL-1β (25 ng/ml), or both IFN-γ (25 ng/ml)/IL-1β (25 ng/ml) for 5 days. The specific activity of ALP (U/μl) was determined as described in the Materials and Methods. Data are representative of three independent experiments performed in triplicate (*p<0.05, Student's t-test).

3)ALP activity in KN-3 cells

We examined the role of proinflammatory cytokines in odontoblastic differentiation of KN-3 cells. The cells were treated with IFN-γ (25 ng/ml) and IL-1β (25 ng/ml) for 5 days, and then subjected to ALP staining. As shown in Fig. 3A, stable expression of ALP was found in untreated KN-3 cells. ALP expression was suppressed in IFN-γ-treated KN-3, but not in IL-1β-treated KN-3 cells. When the cells

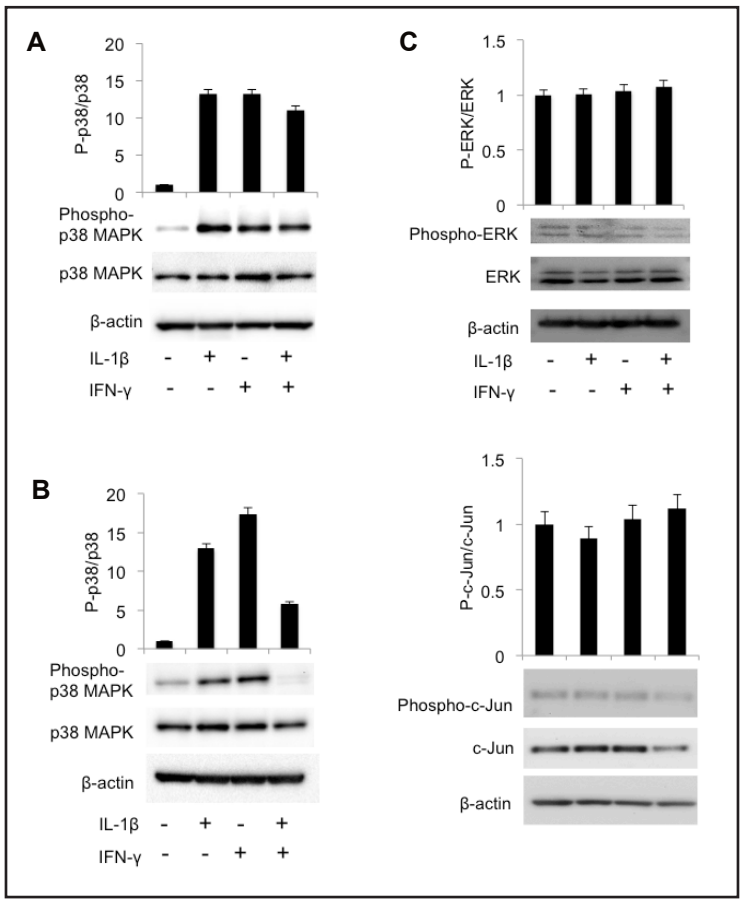

Fig. 4 Phosphorylation of p38 MAPK in KN-3 cells
KN-3 cells were stimulated with IFN-γ (25 ng/ml), IL-1β (25 ng/ml), or both IFN-γ (25 ng/ml)/IL-1β (25 ng/ml) for 1 h (A) and 3 days (B). The expression of p38 and phosphorylated p-38 was determined by immunoblot analysis.
(C)KN-3 cells were stimulated with IFN-γ (25 ng/ml), IL-1β (25 ng/ml), and IFN-γ (25 ng/ml)/IL-1β (25 ng/ml) for 3 days. The expression of ERK, c-jun, phosphorylated ERK, phosphorylated c-jun was determined by immunoblot analysis.

were treated with both IFN-γ and IL-1β, the ALP expression was restored up to the levels of untreated KN-3 cells. We also examined ALP activity using a colorimetric assay. IFN-γ treatment significantly inhibited ALP activity in KN-3 cells compared with that in both untreated and IL-1β-treated KN-3 cells. Restoration of ALP activity was detected in KN-3 cells treated with IFN-γ and IL-1β (Fig. 3B).

4)Expression of phosphorylated p38 MAPK in KN-3 cells

Next, we examined the phosphorylation levels of p38 MAPK in proinflammatory cytokine-treated KN-3 cells. Phosphorylated p38 MAPK was detected in KN-3 cells stimulated with IL-1β, IFN-γ, and both IL-1β/IFN-γ for 1 h. Unphosphorylated p38 MAPK was constantly expressed in KN-3 cells under the same conditions (Fig. 4A). We also examined the expression levels of phosphorylated p38 in KN-3 cells treated with proinflammatory cytokines for 3 days. IFN-γ treatment significantly induced the expression of phosphorylated p38 MAPK in KN-3 cells. However, weak expression of phosphorylation p38 MAPK was detected in KN-3 cells stimulated with IL-1β and IL-1β/IFN-γ for 3 days (Fig. 4B). No change in the expression levels of phosphorylated ERK 1/2 or c-Jun were detected in KN-3 cells stimulated with IL-1β, IFN-γ, or IL-1β/IFN-γ (Fig 4C).

Discussion

It is well known that odontoblasts play a major role in not only dentin formation but also the defense against pathogens. Bacterial pathogens trigger an innate immune response that mainly leads to the secretion of cytokines that facilitate immune reactions and dentinogenesis[3, 20]. However, the physiological and pathological roles of proinflammatory cytokines in the formation of dentin by odontoblasts are still largely unknown. We have previously reported that the odontoblastic differentiation marker DMP-1 is constitutively expressed in KN-3 cells and regulated by lethal heating and BMP-2 treatment *in vitro*, indicating the utility of KN-3 cells to analyze the differentiation of osteoblasts[19, 21]. In the present study, we used KN-3 cells to elucidate the effect of proinflammatory cytokines on DMP-1 expression and ALP activity, and clarified the role of major proinflammatory

cytokines, IFN-γ and IL-1β, in odontoblastic differentiation.

Chronic dental pulp inflammation under dental caries appears to be elicited by bacterial antigens. Among them, *Streptococcus mutans* is known to induce several kinds of cytokines inducing IFN-γ and IL-1β. In particular, Mutans Streptococci in dental shallow caries is thought to induce IFN-γ, which is produced by Th1 cells, followed by orchestration of cell-mediated immune responses in the dental pulp[22, 23]. In early dental carious lesions, the expression of IL-1β gene was not induced[24]. IL-1β production was downregulated by IFN-γ in response to LPS stimulation in murine bone marrow-derived macrophages and dendritic cells[23]. High levels of IL-1β have been detected in inflamed dental pulp *in vivo*, and there are many reports concerning the contribution of proinflammatory cytokines to the induction of dental pulpitis[25].

Piattelli et al. reported that transforming growth factor-β (TGF-β) is secreted by odontoblasts, and its expression is increased in irreversible dental pulpitis to promote dentin mineralization[26]. Interestingly, a recent study reported that TGF-β suppresses the gene expression of DMP-1 in a mouse odontoblast cell line[27], and exerts anti-inflammatory effects through repression of lymphocyte proliferation during the late stages of inflammation[28]. In the present study, we found that IFN-γ suppressed the protein expression of DMP-1 in KN-3 cells. We have no explanation for the precise mechanism, but these results suggest that DMP-1 expression is regulated by inflammatory processes in odontoblasts stimulated with IFN-γ and IL-1β. Further studies will be performed to examine the intracellular signaling involved in IFN-γ-induced inhibition of DMP-1 expression during the inflammatory processes.

Bone and dentin are mineralized tissues that resemble each other in terms of their composition and mechanism of formation[29]. Recent studies have reported crucial effects of proinflammatory cytokines on the suppression of osteoblast development. Young et al reported that IFN-γ produced by activated Th1 cells inhibits ALP activity in primary osteoblasts[30]. On the other hand, IL-1β induces the ALP activity through production of mineralization-related proteins in short-term culture of dental pulp cells. Compared with irreversible pulpitis, some reports have shown that dental pulp tissue expresses high ALP activity during reversible dental pulpitis through dentinogenesis[7, 31, 32]. In addition to these controversial findings, we found that IFN-γ remarkably suppressed DMP-1 expression and ALP activity, and that IL-1β restored the suppression mediated by IFN-γ in KN-3 cells in long-term culture (Fig. 3). These findings suggest that orchestration of proinflammatory cytokines plays a crucial role during the dentin mineralization process of dental pulp in an inflammatory environment.

In vivo experiments have shown high expression of the p38 gene in odontoblasts during primary dentinogenesis, as well as activation of the p38 MAPK pathway during odontoblast stimulation in tertiary dentinogenesis[33]. On the other hand, p38 gene is highly expressed in odontoblast during active primary dentinogenesis and phosphorylated p38 is remarkably downregulated in secondary dentinogenesis[34]. Our results revealed that IFN-γ treatment downregulated DMP-1 protein expression and ALP activity through the enhancement of prolonged phosphorylation p38 MAPK in KN-3 cells for 3 days (Fig. 4). These findings suggest that the phosphorylation of p38 MAPK may affect dentinogenesis through downregulation of DMP-1 and ALP activity. Although the Increase of ALP activity was thought to contribute the differentiation of cells into odontoblasts[35], the upstream cellular mechanism responsible for mediating the expression of ALP in dental pulp remains largely unknown. The p38 pathway is known to be involved in the differentiation of osteoblastic cells by mediating the stimulation of ALP activity[36]. In the present study, we cannot make sure about this issue. Further study is needed to clarify the precise signaling pathways involved in IFN-γ-mediated inhibition of DMP-1 expression through p38 pathway.

In conclusion, this study demonstrated that the proinflammatory cytokines INF-γ and IL-1β regulated the expression of DMP-1 in odontoblast-like cells. IFN-γ treatment downregulated DMP-1 expression and ALP activity, indicating that IFN-γ is critically involved in dentin mineralization of the dental pulp. In addition, IL-1β treatment restored the downregulation of DMP-1 expression and ALP activity through phosphorylation of p38 MAPK. We postulate that the orchestration of proinflammatory cytokines including IFN-γ and IL-1β may have an essential roles in regulation of dentin formation during inflammation of dental pulp. Further investigation is currently in progress to clarify the mechanism by which proinflammatory cytokines regulate dentinogenesis in dental pulp tissue.

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Conflict of interests

None

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