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# Dec2 inhibits macrophage pyroptosis to promote periodontal homeostasis

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## ABSTRACT

**Purpose:** Macrophages play crucial roles as early responders to bacterial pathogens and promote/ or impede chronic inflammation in various tissues. Periodontal macrophage-induced pyroptosis results in physiological and pathological inflammatory responses. The transcription factor Dec2 is involved in regulating immune function and inflammatory processes. To characterize the potential unknown role of Dec2 in the innate immune system, we sought to elucidate the mechanism that may alleviate macrophage pyroptosis in periodontal inflammation.

**Methods:** *Porphyromonas gingivalis* lipopolysaccharide (LPS) was used to induce pyroptosis in RAW 264.7 macrophages. Subsequently, we established an LPS-stimulated Dec2 overexpression cellular model in macrophages. Human chronic periodontitis tissues were employed to evaluate potential changes in inflammatory marker expression and pyroptosis. Finally, the effects of Dec2 deficiency on inflammation and pyroptosis were characterized in a *P. gingivalis*-treated experimental periodontitis Dec2-knockout mouse model.

**Results:** Macrophages treated with LPS revealed significantly increased messenger RNA expression levels of Dec2 and interleukin (IL)-1 $\beta$ . Dec2 overexpression reduced IL-1 $\beta$  expression in macrophages treated with LPS. Overexpression of Dec2 also repressed the cleavage of gasdermin D (GSDMD), and the expression of caspase-11 was concurrently reduced in macrophages treated with LPS. Human chronic periodontitis tissues showed significantly higher gingival inflammation and pyroptosis-related protein expression than non-periodontitis tissues. *In vivo*, *P. gingivalis*-challenged mice exhibited a significant augmentation of F4/80, tumor necrosis factor- $\alpha$ , and IL-1 $\beta$ . Dec2 deficiency markedly induced GSDMD expression in the periodontal ligament of *P. gingivalis*-challenged mice.

**Conclusions:** Our findings indicate that Dec2 deficiency exacerbated *P. gingivalis* LPS-induced periodontal inflammation and GSDMD-mediated pyroptosis. Collectively, our results present novel insights into the molecular functions of macrophage pyroptosis and document an unforeseen role of Dec2 in pyroptosis.

**Keywords:** Dec2; Macrophage; Periodontal inflammation; *Porphyromonas gingivalis*; Pyroptosis

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#### Author Contributions

Conceptualization: Xiaoyan Li, Ujjal K. Bhawal; Formal analysis: Dawei He, Xiaoyan Li, Fengzhu Zhang, Ujjal K. Bhawal; Investigation: Dawei He, Xiaoyan Li, Fengzhu Zhang, Chen Wang, Ujjal K. Bhawal; Methodology: Dawei He, Xiaoyan Li, Fengzhu Zhang, Ujjal K. Bhawal; Project administration: Yi Liu, Ujjal K. Bhawal, Jiang Sun; Writing - original draft: Xiaoyan Li, Ujjal K. Bhawal; Writing - review & editing: Xiaoyan Li, Fengzhu Zhang, Yi Liu, Ujjal K. Bhawal, Jiang Sun.

#### Conflict of Interest

No potential conflict of interest relevant to this article was reported.

## INTRODUCTION

Inflammation is a complex biological phenomenon facilitated by innate and adaptive immune responses against damaged cells and pathogens that is strictly regulated by macrophages, which cause activation of the inflammatory network [1,2]. During periodontal inflammation, pathogens interfere with immune cells to induce inflammatory surveillance with constant alterations in gene expression. The unrestricted production of inflammatory molecules provokes consequential damage to tissues, leading to detrimental effects on the local environment. Macrophages are heterogeneous immune cells with various functions that can aggravate or ameliorate the severity of diseases [3,4]. Macrophages are important innate immune cells that act as sentinels in tissues against lipopolysaccharide (LPS)-induced periodontal inflammation [5]. Macrophage activation leads to the secretion of pro-inflammatory cytokines, such as interleukin (IL)-1 $\beta$ , during early stages of infection [6,7]. In the late stages of inflammation, macrophages undergo pyroptosis, an inflammatory form of programmed cell death [8,9]. LPS is a canonical inducer of the process of pyroptosis [10]. In this context, the interplay between pyroptosis and periodontal inflammation has drawn profound interest.

Pyroptosis enables the elimination of intracellular pathogens [11,12]. Pyroptosis is activated by harmful microorganisms [13] and is dependent on inflammatory caspase [14]. Bacterial and viral infections are the primary causes of intensified macrophage pyroptosis [14]. In recent years, increasing evidence has emerged that macrophage pyroptosis contributes to uncontrolled inflammation in various tissues. As a keystone pathogen in periodontal inflammation, *Porphyromonas gingivalis* [15] regulates innate immunity [16] and may boost macrophage pyroptosis and the secretion of cytokines to mediate inflammation. The release of mediators of inflammation promotes the migration of polymorphonuclear neutrophils (PMNs) to sites of inflammation in the periodontal ligament. Active caspase-1 and caspase-11 are involved in pyroptosis and cleave the cytosolic protein gasdermin D (GSDMD) [14,17]. LPS can activate caspase-11 and stimulate the secretion of IL-1 $\beta$ /IL-18, which subsequently triggers pyroptosis [18].

IL-1 $\beta$  has long been known as a critical pyrogen, and enhanced expression of IL-1 $\beta$  has been found in the gingival tissues of patients with periodontitis [19]. In addition, *P. gingivalis* induces the secretion of IL-1 $\beta$  and subsequent pyroptosis [20]. *P. gingivalis* can persist within macrophages [21,22]. Accordingly, we speculated that *P. gingivalis* might induce periodontal inflammation through GSDMD-mediated pyroptosis. Thus, this study used a mouse model of *P. gingivalis*-challenged periodontal inflammation and cell culture experiments with RAW 264.7 macrophages to imitate the inflammatory response of macrophages in periodontal pyroptosis.

Differentiated embryo chondrocyte 2 (Dec2/Sharp-1/Bhlhe41) is a transcription factor that is involved in immune function and the regulation of inflammatory processes. Dec2 has been shown to regulate Th2 cell differentiation and induce Th2-type cytokine stimulation [23]. These inter-regulatory mechanisms of immune cells, pro-inflammatory cytokines, and pyroptosis can have detrimental effects on periodontal homeostasis and can lead to chronic periodontitis [24]. We recently reported the effects of Dec2 on the activation of inflammasomes and the pyroptosis of gingival and periodontal ligament fibroblasts [25]. However, the definitive role of Dec2 in fundamental processes involved in the activation of periodontal macrophage pyroptosis has not been determined.

Therefore, the aim of this study was to determine whether there is a relationship between Dec2 and LPS-induced inflammatory macrophages that governs GSDMD-dependent pyroptosis. Using an *in vivo* Dec2-knockout (*Dec2*KO) mouse model of periodontal inflammation, we demonstrated that Dec2 might possess bona fide periodontal homeostasis activity, as Dec2-deficient cells exhibited higher levels of inflammatory parameters.

## MATERIALS AND METHODS

### Cellular model

#### Cell culture

The cell line of RAW 264.7 macrophages was purchased from the ATCC (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle medium (Wako, Tokyo, Japan) with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin under 5% CO<sub>2</sub> at 37°C in a humidified environment. The cells were incubated for 24 hours in the presence or absence of 10 µg/mL *P. gingivalis* LPS (LPS-PG Ultrapure, InvivoGen, San Diego, CA, USA) to induce an inflammatory response.

#### Overexpression of Dec2

RAW 264.7 macrophages were seeded at  $1.0\text{--}2.0 \times 10^5$  in 6-well plates and cultured in antibiotic-free normal growth medium. An empty vector or a Dec2 overexpression vector was transfected to the cells using Lipofectamine3000 (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot assays were used to verify the transfection efficacy.

#### RT-PCR

Total RNA was extracted using QIAzol (Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions. A high-capacity complementary DNA (cDNA) archive kit (Applied Biosystems, Foster City, CA, USA) was used for cDNA transcription. TaqMan probes of the target genes, including Dec2 (Mm00470512\_m1), IL-1β (Mm00434228\_m1), and beta-actin (*ACTB*) (Mm02619580\_g1), were used for RT-PCR.

#### Western blots

Whole-cell lysates were collected using radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In this process, 20 µg of protein was separated on precast gels (10% or 15%, Wako, Osaka, Japan) and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk followed by overnight incubation at 4°C with antibodies to caspase-11 (1:500, Abcam, Tokyo, Japan), GSDMD (1:500, Abcam) and glyceraldehyde-3-phosphate dehydrogenase (1:1,000, Cell Signaling Technology, Danvers, MA, USA). Antibodies bound to protein bands were visualized using an ECL Plus Western Blotting Detection System (GE Healthcare, Tokyo, Japan).

### Human gingival tissue model

Gingival tissues were obtained from chronic periodontitis patients and prepared as previously described [26]. The review board of Dalian Stomatological Hospital Committee of Ethics on Human Experiments approved this study (DLKQLL2020025; 05/15/2020). All subjects (9 males, 8 females) signed an informed consent document. The specimens were subjected to antigen retrieval (citrate buffer pH 6.0, Abcam) and peroxidase blocking (Wako, Tokyo, Japan) followed by overnight incubation at 4°C with antibodies to Dec2 (1:100, Novus

Biologicals, LLC, Centennial, CO, USA), CD68 (Nichirei, Tokyo, Japan), GSDMD (1:100, Abcam), F4/80 (1:50, Abcam), PMNs (1:50, Abcam), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; 1:75, Abcam) and IL-1 $\beta$  (1:100, Abcam). The same 3,3'-diaminobenzidine exposure conditions were performed on each specimen after incubation with the secondary antibodies (MAX-PO, Nichirei Biosciences Inc., Tokyo, Japan). Specimens of the upper jaws were stained with tartrate-resistant acid phosphatase (TRAP; Wako, Tokyo, Japan) according to the manufacturer's protocol.

### Mouse periodontitis model

*Dec2*KO (12-week-old, n=12) mice were purchased from Ingenious Targeting Laboratory, Inc. (Stony Brook, NY, USA) [25]. C57BL/6 (WT, 12-week-old, n=12) (CLEA Japan, Inc., Tokyo, Japan) and *Dec2*KO mice were used as periodontitis models. The experimental periodontitis model was established according to our previous study [27]. The mice were divided into a control group and a *P. gingivalis*-treated group and were sacrificed after 30 days. All animal experiments were conducted under the approval of the Animal Ethics Committee of Kanagawa Dental University (approval No. 13-044).

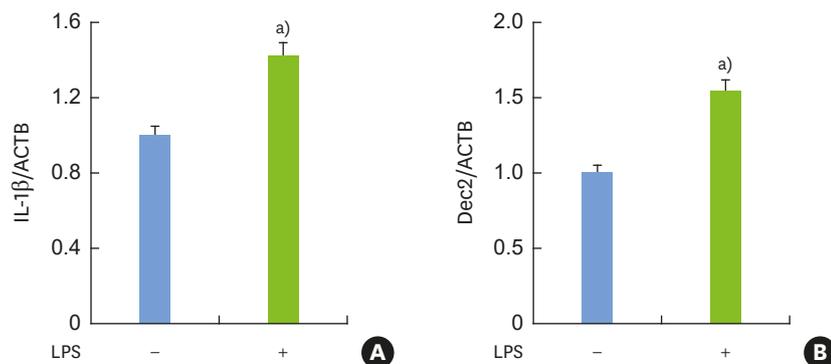
### Statistical analysis

SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The independent 2-tailed Student *t*-test or analysis of variance was performed to assess the results, which were regarded as significantly different if there was a *P*value less than 0.05.

## RESULTS

### The expression of Dec2 in *P. gingivalis* LPS-treated macrophages

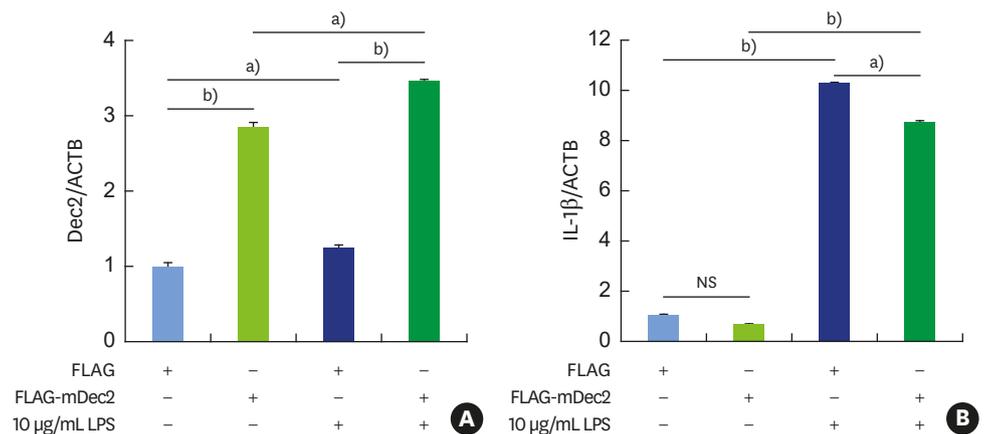
RAW 264.7 macrophages were treated with 10  $\mu$ g/mL *P. gingivalis* LPS for 24 hours, which led to a significant increase in the messenger RNA expression level of IL-1 $\beta$ , as shown by RT-PCR (Figure 1A, *P*<0.05). Interestingly, the expression of the transcription factor Dec2 was also significantly elevated (Figure 1B, *P*<0.05).



**Figure 1.** Reverse transcription-polymerase chain reaction analysis showing that the transcription factor Dec2 is elevated following treatment with *P. gingivalis* LPS for 24 hours. (A) LPS treatment significantly induced mRNA expression of IL-1 $\beta$  in RAW 264.7 macrophages. (B) The mRNA expression level of Dec2 was also significantly elevated. The data shown represent means $\pm$ standard deviation. All results are representative of at least 3 independent experiments.

IL: interleukin, LPS: lipopolysaccharide, mRNA: messenger RNA.

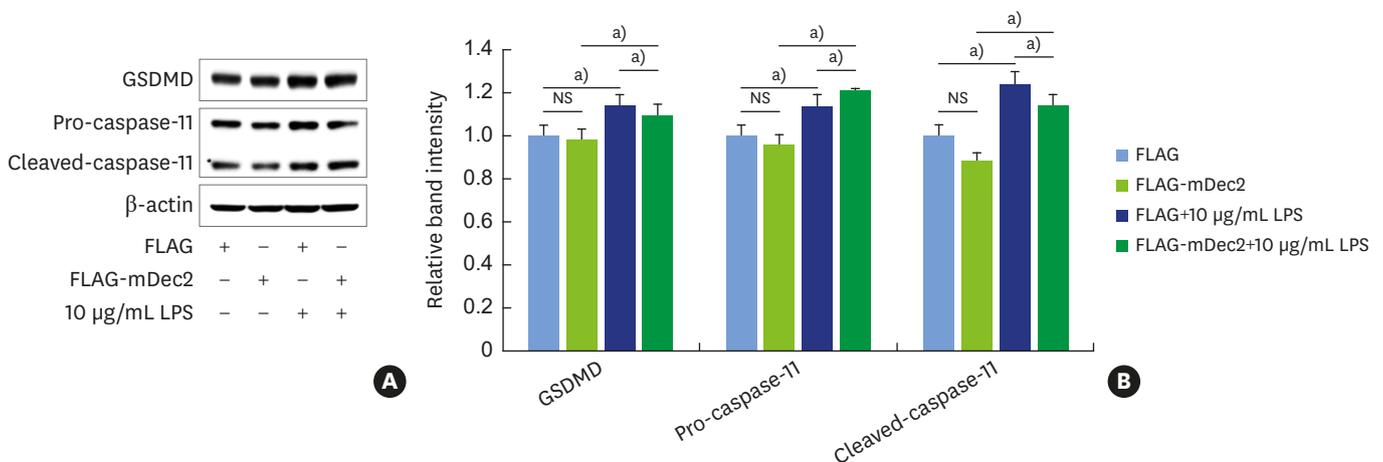
<sup>a)</sup>*P*<0.05.



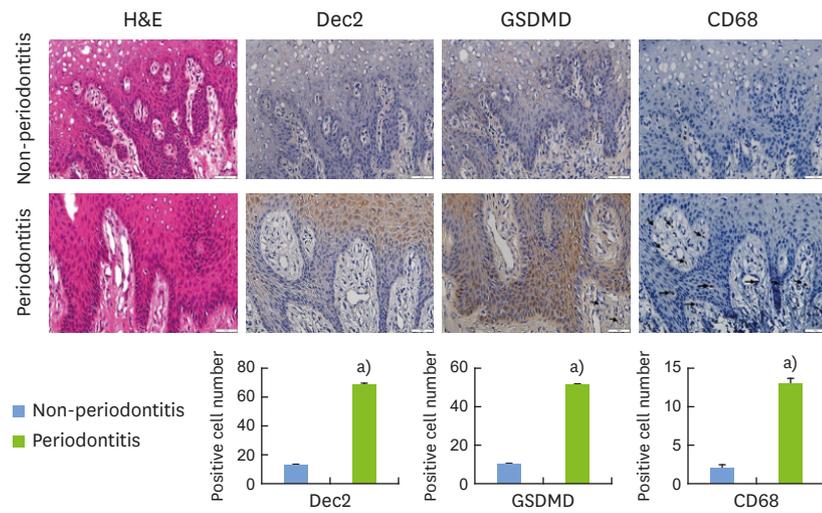
**Figure 2.** Reverse transcription-polymerase chain reaction analysis showing that Dec2 controls *P. gingivalis* LPS-induced pyroptosis. (A) The messenger RNA level of Dec2 was highly upregulated after transfection with the Dec2 expression vector. (B) IL-1β expression was suppressed by the overexpression of Dec2 after treatment with 10 μg/mL *P. gingivalis* LPS. FLAG denotes an empty vector. The data shown represent means±standard deviation. All results are representative of at least 3 independent experiments. IL: interleukin, LPS: lipopolysaccharide, NS: non-significant. <sup>a)</sup> $P < 0.05$ , <sup>b)</sup> $P < 0.01$ .

### The role of Dec2 in *P. gingivalis* LPS-induced pyroptosis

To understand the regulatory role of Dec2 in inflammation mediated by treatment with *P. gingivalis* LPS, cell pyroptosis was assessed. The overexpression of Dec2 in RAW 264.7 macrophages was confirmed by RT-PCR (Figure 2A,  $P < 0.05$ ,  $P < 0.01$ ), followed by the suppression of IL-1β expression, after treatment with 10 μg/mL *P. gingivalis* LPS (Figure 2B,  $P < 0.05$ ,  $P < 0.01$ ). Further, treatment with 10 μg/mL *P. gingivalis* LPS led to a significant induction of GSDMD (Figure 3A and B,  $P < 0.05$ ) and cleaved caspase-11 (Figure 3A and B,  $P < 0.05$ ). The overexpression of Dec2 alleviated the expression of cell pyroptosis markers (Figure 3A and B,  $P < 0.05$ ) and controlled cell pyroptosis under inflammatory conditions. These results revealed the inhibitory effect of Dec2 on LPS-induced cell pyroptosis.



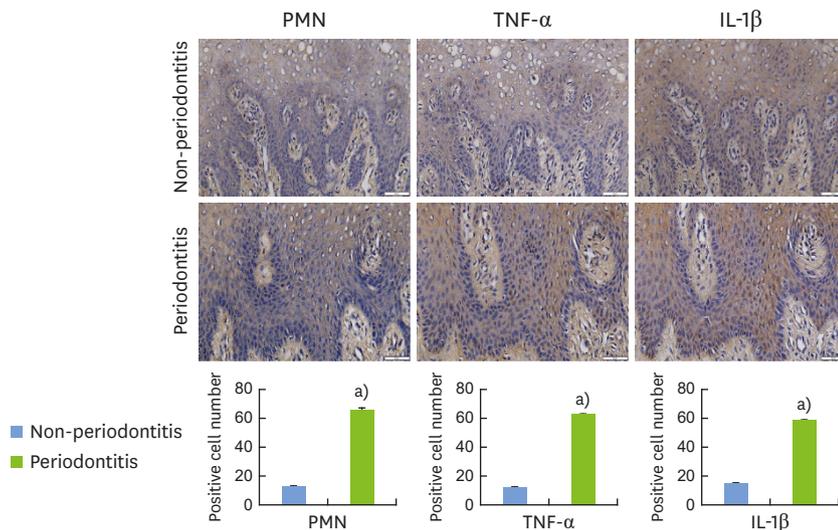
**Figure 3.** Western blot analysis showing that Dec2 reduces the *P. gingivalis* LPS-induced pyroptosis. (A, B) Treatment with 10 μg/mL *P. gingivalis* LPS led to significant increases of GSDMD and pro- and cleaved caspase-11. The overexpression of Dec2 alleviated the expression of cell pyroptosis markers and controlled cell pyroptosis under inflammatory conditions. FLAG denotes an empty vector. The data shown represent means±standard deviation. All results are representative of at least 3 independent experiments. GSDMD: gasdermin D, LPS: lipopolysaccharide, NS: non-significant. <sup>a)</sup> $P < 0.05$ .



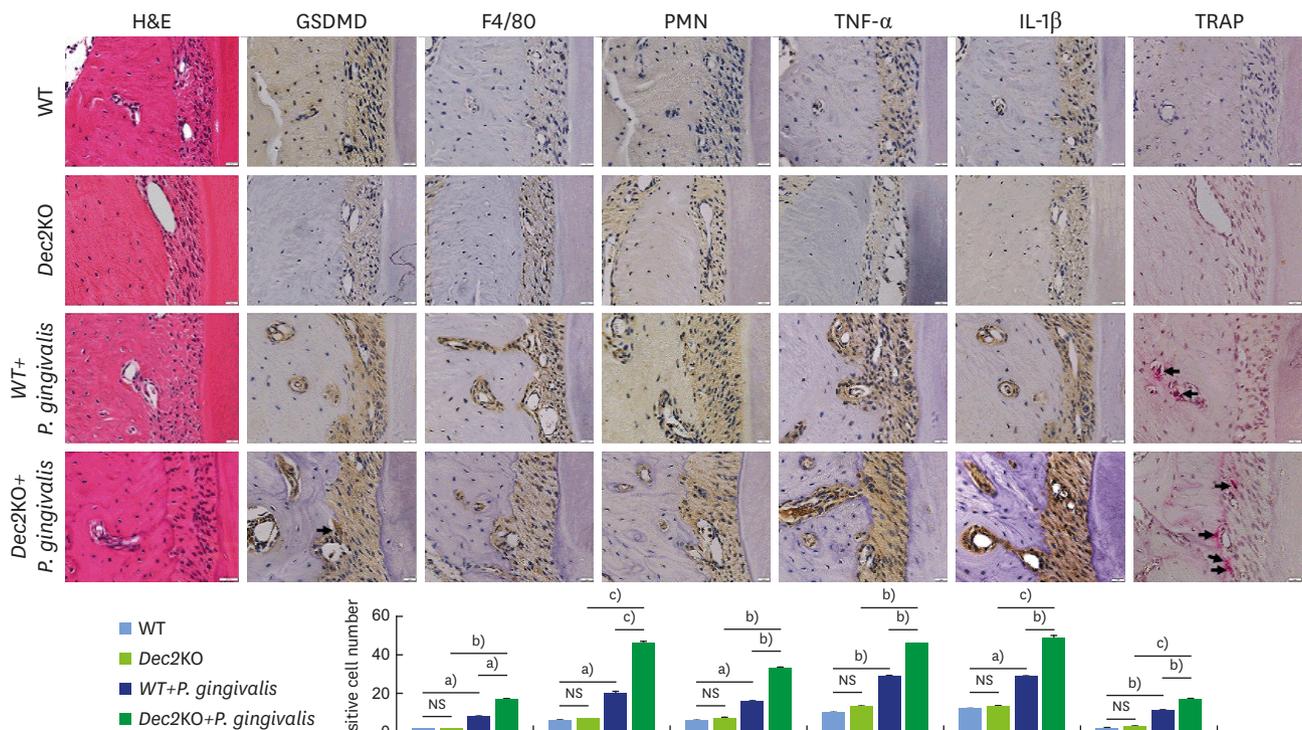
**Figure 4.** Immunohistochemistry showing that Dec2 is upregulated in chronic periodontitis tissues. The expression of Dec2 was higher in periodontitis tissue than in non-periodontitis tissue. The expression of Dec2, GSDMD, and CD68 was also consistently induced in periodontitis tissues. The data shown represent means±standard deviation. All results are representative of at least 3 independent experiments. H&E: hematoxylin and eosin, GSDMD: gasdermin D. <sup>a)</sup> $P<0.001$ .

### The expression of Dec2 in human gingival tissue with chronic periodontitis

The expression level of the Dec2 protein was significantly higher in chronic periodontitis tissues than in non-periodontitis healthy tissues. A consistent pattern of significantly higher expression levels of Dec2 (Figure 4,  $P<0.001$ ), GSDMD (Figure 4,  $P<0.001$ ), CD68 (Figure 4,  $P<0.001$ ), PMNs (Figure 5,  $P<0.001$ ), TNF- $\alpha$  (Figure 5,  $P<0.001$ ), and IL-1 $\beta$  (Figure 5,  $P<0.001$ ) was also found in the periodontitis tissues.



**Figure 5.** Immunohistochemistry showing that inflammation is induced in chronic periodontitis tissue. The expression levels of PMNs, TNF- $\alpha$ , and IL-1 $\beta$  were significantly elevated in chronic periodontitis tissue. The data shown represent means±standard deviation. All results are representative of at least 3 independent experiments. PMN: polymorphonuclear neutrophil, TNF- $\alpha$ : tumor necrosis factor- $\alpha$ , IL-1 $\beta$ : interleukin-1 $\beta$ . <sup>a)</sup> $P<0.001$ .



**Figure 6.** Immunohistochemistry showing that a deficiency of Dec2 facilitates the infiltration of inflammatory cells and osteoclast differentiation in periodontitis models. The expression of GSDMD was upregulated in *Dec2KO* mice compared with WT mice, and consistently, the infiltration of inflammatory cells was increased more in *Dec2KO* mice compared with WT mice, as well as the inflammatory markers, which included IL-1 $\beta$  and TNF- $\alpha$ . Notably, a higher number of TRAP-positive osteoclasts was detected in *Dec2KO* mice than in WT mice; black arrows indicate TRAP-positive cells. The data shown represent means $\pm$ standard deviation. All results are representative of at least 3 independent experiments.

H&E: hematoxylin and eosin, GSDMD: gasdermin D, PMN: polymorphonuclear neutrophil, *Dec2KO*: Dec2-knockout, WT: wild-type, TNF- $\alpha$ : tumor necrosis factor- $\alpha$ , IL-1 $\beta$ : interleukin-1 $\beta$ , TRAP: tartrate-resistant acid phosphatase, NS: not significant.

<sup>a)</sup> $P < 0.05$ , <sup>b)</sup> $P < 0.01$ , <sup>c)</sup> $P < 0.001$ .

### The infiltration of inflammatory cells in a Dec2 deficient mouse periodontitis model

To verify the inflammation-inhibiting role of Dec2, a periodontitis model was used. The expression of GSDMD (Figure 6,  $P < 0.05$ ,  $P < 0.01$ ) was significantly upregulated in *Dec2KO* mice compared to WT mice, and consistently, the infiltration of inflammatory cells (Figure 6,  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ ) was higher in *Dec2KO* mice than in WT mice, as well as inflammatory markers, including IL-1 $\beta$  (Figure 6,  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ ) and TNF- $\alpha$  (Figure 6,  $P < 0.01$ ). Notably, we also detected a higher number of TRAP-positive cells in *Dec2KO* mice than in WT mice (Figure 6,  $P < 0.01$ ,  $P < 0.001$ ).

## DISCUSSION

This study demonstrated that Dec2 overexpression inhibits pyroptosis in LPS-treated macrophages. We observed that overexpression of Dec2 inhibited the expression of GSDMD, IL-1 $\beta$ , and pro- and cleaved caspase-11; these findings demonstrate that Dec2 blocks inflammation-triggered pyroptosis. Both caspase-1 and caspase-11 play roles in the innate host defense mechanism against inflammation since caspase-11 predominantly contributes to the response to bacterial infections [28]. Several studies have reported that pyroptosis, a novel inflammatory form of programmed cell death, involves the caspase-1 and/or caspase-11

dependent cleavage of IL-1 $\beta$  [29]. Our results suggest that LPS aids the inflammatory response in macrophages and that Dec2 attenuates LPS-induced pyroptosis *in vitro*.

We used RAW 264.7 macrophages as our *in vitro* model to gain insights into the cellular effects of Dec2 in the LPS-induced pyroptosis of macrophages. Under normal conditions, there is a low abundance of resting macrophages, which have a slow metabolism and long-term survival [30]. During periodontal inflammation, the gingiva-resident macrophages recruit monocytes/macrophages from periodontal tissues to the inflammation area to maintain tissue homeostasis. Hence, it is important to use RAW 264.7 macrophages to study the inflammatory response *in vitro*. We explored the inflammatory activation of macrophages in *Dec2*KO mice, where they affect the tissue environment concomitantly with pyroptosis.

Pyroptosis, a recently identified type of programmed and pro-inflammatory cell death, allows the removal of intracellular bacterial pathogens to overcome the microbial load [31]. Pyroptosis is initiated by inflammasomes and is executed by GSDMD [32], through a process in which cleaved GSDMD induces pyroptosis by inflammatory caspases-1 and -11 [33]. Caspase-1 is activated upon inflammasome activation, while caspase-11 activation responds to bacterial LPS, and subsequently provokes pyroptosis [34]. GSDMD deletion has consistently alleviated inflammation-driven diseases in various mouse models [35,36]. An enhanced accumulation of pyroptotic cells and increased GSDMD expression were observed following treatment with LPS [37]. In this study, Dec2-overexpressing macrophages treated with LPS had substantially reduced expression levels of the GSDMD protein in comparison to untreated cells. The *Dec2*KO mouse model reversed this tendency, suggesting the presence of macrophage pyroptosis in periodontal inflammation and showing that Dec2 plays a crucial role in promoting GSDMD-activated macrophage pyroptosis.

Bacterial pathogens quietly evade the host immune responses due to their long-term self-renewal capacity [38]. Treatment with *P. gingivalis* LPS stimulates inflammasome activation and the consequent GSDMD-triggered macrophage pyroptosis. LPS treatment activates a variety of pathways leading to a dramatic inflammatory response [39]. The transcription level of the pro-inflammatory cytokine IL-1 $\beta$  significantly increased after LPS treatment of macrophages in our study.

Several danger signals stimulate expression of the salient pro-inflammatory mediator IL-1 $\beta$  in various diseases [40]. The levels of IL-1 $\beta$  are higher in patients with periodontitis than in healthy subjects [20]. The representative periodontal pathogenic bacterium *P. gingivalis* induces periodontal pyroptosis [25]. Our *in vivo* data suggest that treatment with *P. gingivalis* LPS substantially enhances periodontitis in the context of induced expression of cleaved caspase-11 and IL-1 $\beta$ . The same trend was evident in our *in vitro* study, in which GSDMD and cleaved caspase-11 were upregulated following LPS treatment. We demonstrated that Dec2 activation by LPS ameliorated both GSDMD-dependent pyroptosis as well as mature IL-1 $\beta$  levels in macrophages. Our findings illustrate the critical role of macrophage responses to *P. gingivalis* LPS in periodontal pyroptosis and demonstrate the unique role of *P. gingivalis* LPS in host-pathogen crosstalk as a regulator of pyroptosis.

In conclusion, these findings show that Dec2 overexpression reduces levels of IL-1 $\beta$  and sequentially regulates caspase-11 and GSDMD, thereby inhibiting pyroptosis. The upregulation of GSDMD is caused, at least in part, by the Dec2 deficiency in LPS-stimulated RAW 264.7 macrophages; this finding provides important mechanistic details regarding the

involvement of Dec2 in the context of inflammation and GSDMD activation in macrophages. Finally, by generating *Dec2*KO mice and analyzing their responses to *P. gingivalis*-induced periodontal inflammation, we demonstrated that Dec2 deficiency enhances the activation of GSDMD and promotes pyroptosis *in vivo*. Our results advance our scientific understanding of periodontal pathogenesis and should lead to the design of innovative approaches for this debilitating condition.

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