ORIGINAL ARTICLE

Mixed lineage kinase domain-like pseudokinase-mediated necroptosis aggravates periodontitis progression

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Abstract
Necroptosis is a form of cell death that is reportedly involved in the pathogenesis of periodontitis. The role of Mlkl-involved necroptosis remains unclear. Herein, this project aimed to explore the role of MLKL-mediated necroptosis in periodontitis in vitro and in vivo. Expression of RIPK3, MLKL, and phosphorylated MLKL was observed in gingival tissues obtained from healthy subjects or patients with periodontitis. The cell viability of Porphyromonas gingivalis lipopolysaccharide (LPS-Pg)-treated cells was detected. In wild type or Mlkl deficiency mice with ligature-induced periodontitis, alveolar bone loss and osteoclast activation were assessed. mRNA levels of inflammatory cytokines in bone marrow-derived macrophages were tested by qRT-PCR. Increased expression of RIPK3, MLKL, and phosphorylated MLKL was observed in gingival tissues obtained from patients with periodontitis. Porphyromonas gingivalis lipopolysaccharide (LPS-Pg)-treated cells developed necroptosis after caspase inhibition and negatively regulated the NF-κB signaling pathway. In mice with ligature-induced periodontitis, Mlkl deficiency reduced alveolar bone loss and weakened osteoclast activation. Furthermore, genetic ablation of Mlkl in LPS-Pg-treated bone marrow-derived macrophages increased the mRNA levels of tumor necrosis factor-α, interleukin (II)-1β, II-6, cyclooxygenase 2, matrix metalloproteinase 9, and receptor activator of nuclear factor kappa-B ligand. Our data indicated that MLKL-mediated necroptosis aggravates the development of periodontitis in a Mlkl-deficient mouse. This will provide a new sight for the understanding of etiology and therapies of periodontitis.

Key messages
- MLKL expression was up-regulated in inflamed human gingival tissue.
- Mlkl deficiency affected the progression of periodontitis.
- Necroptosis played a major role in mice periodontitis model.
- Knockout of Mlkl had a significant effect on inflammatory responses.

Keywords
Necroptosis · Periodontitis · Cell death · Alveolar bone loss · Inflammation

Introduction
Periodontitis, a chronic inflammatory disease induced by a pathogenic periodontal microbiome, manifests the destruction of the periodontium. The prevalence of periodontitis is as high as 45 to 50%; the severe periodontitis affects 11.2% of the world’s population [1]. It is one of the major oral diseases and affects about half of the adult population worldwide [2]. Moreover, periodontitis is a risk factor for many systemic conditions, including cardiovascular disease, lung disease, preterm labor, and diabetes mellitus [3, 4]. Periodontal homeostasis is achieved by intricate regulatory mechanisms functioning in concert [5]. For example, cell proliferation and cell death are two essential processes in maintaining homeostasis in metazoans, and disturbances in these processes may lead to disease development.

Currently, apoptosis, necroptosis, autophagy, and pyroptosis are recognized forms of cell death [6]. Recent studies have demonstrated that a failure to regulate cell death may be involved in the pathogenesis of inflammatory diseases [7–9]. Apoptosis is supposed as “self-killing” that mediated by caspase-dependent pathways. Apoptosis is typically characterized by membrane blebbing, DNA fragmentation, and caspase activation. Autophagy is mediated by caspase-independent pathways resulting in cell degradation.
in contrast to apoptosis. Typically, pyroptosis is regulated by the caspase-1-dependent signaling pathway. However, caspase-1 is not involved in apoptosis or autophagy. During necroptosis, immunostimulants are released inside the cell, and therefore, inflammation is an important pathological feature of necroptosis [10–12]. Although some studies have shown that the pathogenesis of periodontitis is related to autophagy or apoptosis, little is known about the link between necroptosis and periodontitis [13–15].

Necroptosis has been implicated in the pathogenesis of chronic inflammatory diseases, such as inflammatory bowel disease (IBD) [16]. At the molecular level, receptor-interacting protein kinase 1 (RIPK1) plays a crucial role in mediating caspase-independent necroptosis, which is suppressed by a small-molecule inhibitor of RIPK1, necrostatin-1 (Nec-1) [17]. Experimentally, caspase-independent necroptosis can be initiated by a pan-caspase inhibitor, such as zVAD-fluoromethyl ketone (zVAD) [18]. Receptor-interacting protein kinase 3 (RIPK3) is a key driver of necroptosis and is induced by cell death triggers, such as cytokines (for example, tumor necrosis factor receptor 1) [19, 20], toll-like receptors (TLRs) [21, 22], and intracellular RNA and DNA sensors [23, 24]. RIPK3 activates the downstream effector mixed lineage kinase domain-like pseudokinase (MLKL) in necroptosis [16, 25]. Phosphorylation of MLKL (p-MLKL) by RIPK3 induces its oligomerization. The oligomer is then transferred to the plasma membrane where it interacts with phosphatidylinositol, penetrates the cell membrane, and causes cell death [17]. At present, Mlkl-deficient mice are considered to be the best tool for investigating necroptosis [26].

Recently, several reports have shown that necroptosis is involved in the pathogenesis of periodontitis [27–29]. In this study, MLKL-mediated necroptosis was explored both in vitro and in vivo. These findings could provide valuable insights into the role of necroptosis in the pathogenesis of periodontitis and add to our current understanding of periodontitis.

Materials and methods

Ethics statement

This study, including the method of obtaining consent, was approved by the Clinical Ethics Committee of the School of Stomatolgy, Tongji University, in accordance with government-issued guidelines and institution policies (Approval number 2018012). Written informed consent was obtained from all the participants involved in this study. All subjects were over 18 years of age and were assessed for the presence/history of periodontal disease. Smokers, pregnant women, and nursing women were excluded from the study. Probing depth, clinical attachment loss, bleeding on probing, and physiological bone loss were assessed. The periodontitis group consisted of subjects with severe periodontitis, but without other serious systemic conditions. The healthy group consisted of volunteers in general good health who showed no signs of clinical attachment loss, no physiological bone loss with probing depth ≤3 mm, instances of bleeding on probing were <10%, and there was no visible gingival inflammation [30]. A gingival biopsy was collected from each subject (4 per group).

Mice

Specific pathogen-free male mice were used in this study. Mlkl-deficient (Mlkl−/−) mice shared a common genetic C57BL/6 background with wild-type (WT) mice. Animal experiments were conducted in accordance with the guidelines of the Clinical Ethics Committee at the School of Stomatolgy, Tongji University (Approval number 2018011).

Cell culture

WT and Mlkl−/− mice, aged 6–8 weeks, were sacrificed by cervical dislocation. The mice were sanitized by immersing them in 75% alcohol for 2 min. To harvest and culture bone marrow-derived macrophages (BMDMs), the intact femur and tibia were removed and placed in phosphate-buffered saline solution. Both ends of the bone were then removed, and the bone marrow cavity was rinsed with Dulbecco’s modified Eagle’s medium (DMEM) until the cavity turned white. Cells were harvested at room temperature, centrifuged, and resuspended in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20 ng/mL macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA). L929 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Western blots

Gingival tissues of healthy individuals and patients with periodontitis were obtained. Frozen gingival tissues were homogenized rapidly in liquid nitrogen and lysed on ice in a lysis buffer comprised of 50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, sodium orthovanadate, sodium fluoride, EDTA, and leupeptin, supplemented with 1% protease inhibitor cocktail (Bimake, Houston, TX, USA). After 30 min, the lysates were centrifuged at 12,000 g for 15 min at 4 °C. The protein concentration was determined using the bicinchoninic acid method (Beyotime, Shanghai, China). Twenty micrograms of total protein were separated...
on an 8% sodium dodecyl sulfate polyacrylamide gel via electrophoresis, then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). After complete protein transfer, the membranes were blocked with 5% milk powder solution for 2 h at room temperature and incubated overnight at 4 °C with the following rabbit monoclonal anti-RIPK3 (Abcam, Shanghai, China), anti-MLKL (Proteintech, Rosemont, IL, USA),

| Gene         | Forward (5’ to 3’)                      | Reverse (5’ to 3’)                      |
|--------------|----------------------------------------|----------------------------------------|
| Human gapdh  | TGTGTCGGCTGGATCTGTA                    | CCTGCTTACCACTTTCTTTGA                  |
| Human Ripk3  | CAGTGTCACAGCCAGCAGAC                   | CAGTGTCACAGCCAGCAGAC                   |
| Human Mlkl   | TGAGGCACTTTGTGAATCGT                  | GGTGAGCCATCTTCACATT                    |
| Gapdh        | TGCACACATGCATGCCATC                    | GGAGCACAATTTTCCTGAG                    |
| Tnf-α        | CCCTCACACTACATCTTCCT                  | GCTAGCGTGGGCTACAG                      |
| Il-6         | TTGCGCAATGCAATTTCTGAT                 | CTCTAGAGAATCCTGGCTTTTG                 |
| Mmp9         | GCTGACTAGATAAGGACGGCA                  | GCGGCCCTCAAGATGAACGG                   |
| Mmp9         | GCTGACTAGATAAGGACGGCA                  | GCTGACTAGATAAGGACGGCA                  |
| Rankl        | GCTGACTAGATAAGGACGGCA                  | GCTGACTAGATAAGGACGGCA                  |

Table 1 Primer sequence for qRT-PCR

Fig. 1 Necroptosis is involved in the pathogenesis of periodontitis. A Western blots of RIPK3, MLKL, and p-MLKL proteins in gingival tissues from healthy subjects and periodontitis patients (both n = 4). B Relative protein levels after normalization to GAPDH. C Ripk3 and Mlkl mRNA levels normalized to human gapdh expression in gingival tissues from healthy subjects and periodontitis patients. Abbreviations: H healthy subjects, P periodontitis patients, RIPK3 receptor interacting protein kinase-3, MLKL mixed lineage kinase domain-like pseudokinase, p-MLKL phosphorylated MLKL.
anti-phospho-MLKL (p-MLKL) (Abcam), or anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA). Immunodetection was performed using the Odyssey CLx (LI-COR, Lincoln, NE, USA), and the blots were quantified using ImageJ. Treated L929 cells similarly subjected to western blots.

**Cell viability assay**

Cells were seeded into 96-well plates and treated with 1 µg/mL *Porphyromonas gingivalis* lipopolysaccharide (LPS-Pg; InvivoGen, San Diego, CA, USA); 20 µM of the pan-caspase inhibitor, zVAD (Promega, Madison, WI, USA); or 30 µM Nec-1 (Cambridge Bioscience, Cambridge, UK) for 24 h. Cell survival was determined using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA). Luminescence was read using an Infinite M200 microplate reader (Tecan, Zürich, Switzerland).

**RNA extraction, reverse transcription, and the quantitative real-time polymerase chain reaction**

Total RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA, USA). The quantity of RNA was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham MA, USA). One microgram of RNA was used for reverse transcription using the RT reagent kit (Takara, Kusatsu, Japan). The qRT-PCR was performed using SYBR Green PCR Master Mix in a LightCycler 480® (Roche, Basel, Switzerland). Fold change in mRNA levels was calculated by the Eq. \(2^{-\Delta\Delta C_t}\) method [31]. Primer sequences are shown in Table 1.

**Ligature-induced experimental periodontitis**

The experimental periodontitis model was induced in WT and *Mlkl*−/− mice at eight weeks of age (ten per group). A 5–0 silk ligature was tied around the maxillary right second molar, while the contralateral unligated tooth served as the baseline control. Alveolar bone loss (ABL) was analyzed 7 days after ligature placement. For this, the distance between the cementoenamel junction and the alveolar bone crest (CEJ–ABC distance) was measured on both the buccal and palatal sides. Further, the maxillae of the mice were subjected to micro-computed tomography (CT) and histological analyses. Mice in which the ligatures were lost were excluded from the analyses.

![Fig. 2](image-url) LPS-Pg induces necroptosis in a caspase-independent manner and negatively regulates the NF-κB signaling pathway. **A** Viability of BMDM cells treated with 1 µg/mL LPS-Pg, zVAD 20 µM, or Nec-1 30 µM for 24 h. **B** Viability of L929 cells under above exposure conditions. **C** Western blots of p65, phosphorylated (p)-p65, JNK, and p-JNK proteins in L929 cells treated with above exposures for 24 h. **D** Viability of L929 cells pretreated with a p65 or JNK inhibitor for 1 h and then treated with 1 µg/mL LPS-Pg and zVAD 20 µM for 24 h. Data representative of three separate experiments. Values are expressed as means ± SEM. Abbreviations: LPS-Pg *Porphyromonas gingivalis* lipopolysaccharide, Nec-1 necrostatin-1, PDTC p65 inhibitor, SP600125 JNK inhibitor, ns statistically non-significant difference (\(P > 0.05\)); ****\(P < 0.0001\); ***\(P < 0.001\)
Bone loss measurements

For micro-CT analysis, the maxillae were fixed in 4% paraformaldehyde until analysis. To calculate the ABL, the CEJ–ABC distance on the control tooth was subtracted from that on the ligated tooth.

Histological analyses

For histological analyses, the maxillae were fixed in 4% paraformaldehyde, decalculated in 10% EDTA solution for 3 weeks, and then embedded in paraffin. Sections were stained with hematoxylin and eosin and tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated (> 3 nuclei) cells were counted as osteoclasts.

Statistical analysis

Statistical significance between the groups was determined using the two-tailed Student’s t-test or one-way ANOVA. All analyses were performed using Prism 7.0 software (GraphPad, La Jolla, CA, USA). \( P < 0.05 \) was considered significant.
Results

Necroptosis is involved in periodontitis

The upregulation of proteins belonging to the necroptosis machinery, such as RIPK3 and, more specifically, MLKL and p-MLKL, is a strong indication of necroptosis [26]. We evaluated protein and transcript levels of RIPK3, MLKL, and p-MLKL in gingival tissues from healthy subjects and patients with periodontitis using western blots and qRT-PCR. In periodontitis patients, RIPK3, MLKL, and p-MLKL protein levels increased dramatically compared to those in healthy patients (Fig. 1A, B). Similarly, Ripk3 and Mlkl mRNA levels also increased in patients with periodontitis compared to those in healthy subjects (Fig. 1C). Collectively, these results suggest that necroptosis is activated in periodontitis.

LPS-Pg did not induce cell death but combined with zVAD activates necroptosis and negatively regulates the NF-κB signaling pathway

Necroptosis can be activated via distinct pathways, including TLR4-mediated signaling [32]. P. gingivalis is a key pathogen that responsible for periodontitis, and LPS derived from P. gingivalis is known to act as an activator of TLR4 [33]. In this study, BMDMs from WT mice, and L929 cells, were treated with the pan-caspase inhibitor, zVAD in conjunction with LPS-Pg or Nec-1 (a RIPK1 inhibitor) for 24 h. Interestingly, exposure to LPS-Pg alone was not sufficient to induce necroptosis in either BMDMs or L929 cells. Cells co-incubated with zVAD and LPS-Pg exhibited necroptosis, while incubation with Nec-1 reversed this effect, indicating that LPS-Pg treated cells exhibit caspase-independent necroptosis (Fig. 2A, B). The expression of proteins involved in the mitogen-activated protein kinase/c-jun N-terminal kinase and NF-κB signaling pathways were detected in L929 cells. Thus, the survival rates of L929 cells treated with the corresponding inhibitors of these pathways were assessed (Fig. 2C, D). Protein levels of p65 and p-p65 decreased in cells treated with LPS-Pg and zVAD, leading to the activation of necroptosis. On the other hand, p65 inhibitor further sensitized L929 cells to LPS-Pg and zVAD stimulation, leading to a more profound activation of necroptosis. This suggests that activation of the NF-κB signaling pathway prevents LPS-Pg-mediated necroptosis.

Mlkl deficiency mitigates alveolar bone loss in mice with ligature-induced periodontitis

To validate the role of MLKL-mediated necroptosis in the pathogenesis of periodontitis, an experimental model
of periodontitis was established in WT and Mlkl−/− mice. Micro-CT scans of ligated teeth revealed significantly more alveolar bone resorption in WT compared to Mlkl−/− mice (Fig. 3A). Although the CEJ-ABC distances were different between the two groups (Fig. 3B), the buccal side, especially sites corresponding to cusps, presented milder alveolar bone loss in Mlkl−/− compared with WT mice (Fig. 3C). Collectively, these results indicate that Mlkl deficiency protects mice against periodontitis.

Mlkl deficiency weakens the osteoclast activation in mice subjected to ligature-induced periodontitis

TRAP staining demonstrated that the number of osteoclasts increased in the ligation model compared with that in the control (Fig. 4A). However, the number of osteoclasts decreased in Mlkl−/− compared with WT mice (Fig. 4B). Hematoxylin and eosin staining revealed an infiltration of inflammatory cells, a loss of connective tissue attachment, and a resorption of the alveolar bone in periodontal tissue. Inflammation and alveolar bone loss were observed in both WT and Mlkl−/− mice (Fig. 4C). These results suggest that Mlkl deficiency weakens osteoclast activation in mice with ligature-induced periodontitis.

LPS-Pg induces differential expression of inflammatory cytokine- and osteoclast-related genes in BMDMs from WT and Mlkl−/− mice

To get further insight into the impact of Mlkl in regulating the expression of cytokine- and osteoclast-related genes, BMDMs from WT and Mlkl−/− mice were cultured and stimulated with LPS-Pg for different periods of time. Transcript levels of the inflammatory cytokine genes, tumor necrosis factor-alpha, interleukin (II)-1β, cyclooxygenase 2, and II-6, (Fig. 5A), and of the osteoclast-related genes, matrix metalloproteinase 9 and receptor activator of nuclear factor kappa-B ligand, were measured by qRT-PCR. A significant difference was observed in expression of inflammatory cytokine and osteoclast-related genes between BMDMs from WT and Mlkl−/− mice (Fig. 5B). In the absence of LPS-Pg treatment, a difference in expression of the aforementioned genes was not found. But expression of the cytokine genes in BMDMs from Mlkl−/− mice was higher than WT mice at the early 4-h
time point; these effects were reversed later. While in the case of Tnf-α, the levels were neither significantly different at 12 or 24 h. Similar to the expression of cytokine genes, osteoclast-related genes showed a decrease in expression in BMDMs from Mlkl−/− compared to WT mice. Altogether, our data demonstrate that MLKL-mediated necroptosis is required for LPS-induced expression of cytokine- and osteoclast-related genes.

**Discussion**

Periodontitis is an inflammatory response of the host’s immune system against periodontal pathogenic bacteria in an attempt to clear the bacterial invasion; however, this occurs at the cost of a loss of periodontal tissue. In this context, cell proliferation and cell death are two essential elements maintaining homeostasis in periodontal tissue [6]. Recently, necroptosis was identified as a form of cell death that occurs during infection and sterile inflammation. Increased expression of RIPK3 and MLKL is an indicator of necroptosis [26]. Both RIPK3- and MLKL-dependent necroptosis have been reported to be involved in systemic inflammatory conditions such as accelerating tissue injury in acute pancreatitis (He et al. 2009). Ke et al. reported that *P. gingivalis* may cause necroptosis during the development of periodontitis [28]. Phosphorylated (p)-MLKL is considered a specific marker of necroptosis [34]. When we analyzed gingival biopsies from healthy subjects and patients with periodontitis, it was found that RIPK3, MLKL, and p-MLKL protein levels were significantly higher in the latter. These results corroborate the finding that necroptosis occurs in periodontitis. However, whether the upregulation of RIPK3 and/or MLKL serves as a primary causal factor or is a secondary consequence of persistent inflammation remains unclear and needs to be further studied [8].

Kondylis et al. reported that the IKK/NF-κB signaling pathway inhibits RIPK1 activity-dependent cell death, thus protecting the host against a homeostatic imbalance and inflammation in the intestine [35]. Activation of the NF-κB signaling pathway promotes cell survival, cell proliferation, and the production of pro-inflammatory cytokines [17]. This is confirmed by our finding that LPS-Pg-treated cells, incubated with a caspase inhibitor, developed necroptosis, and exhibited negative regulation of the NF-κB signaling pathway. Necroptosis triggers the release of pro-inflammatory cytokines, including tumor necrosis factor and interferon, which are potent inducers of necroptosis, further prolonging inflammation [8]. MLKL is less pleiotropic than RIPK1 and RIPK3. Thus, Mlkl−/− mice are preferred for investigating necroptosis in the experimental periodontitis model used in the present study [26]. Differential expression of inflammatory cytokine- and osteoclast-related genes was observed in WT and Mlkl−/− mice. Here, we showed that Mlkl deficiency suppressed Il-6 and Cox2 expression evoked by LPS-Pg at 12 h and 24 h compared to 4 h. It is difficult to conclude from our data the reason of expression difference on cytokines. These results might suggest that LPS-Pg sensitizes Mlkl-deficient BMDMs to produce pro-inflammatory cytokines. The ability to release inflammatory cytokines is moderated due to the absence of MLKL-mediated necroptosis. However, it is interesting that Wu J. et al. shown there was no statistically significant difference between WT and Mlkl-deficient BMDMs in cytokine production response to LPS [36]. ABL and osteoclast activation were suppressed in Mlkl−/− compared with WT mice, suggesting that Mlkl deficiency mitigated inflammation.

In conclusion, MLKL mediates necroptosis in inflamed periodontal tissues. Previous studies investigating the relationship between periodontitis and necroptosis have focused on cellular or WT animal models [27, 28]. Our study is the first to validate the fact that necroptosis aggravates the development of periodontitis in a Mlkl-deficient mouse model. However, how necroptosis interacts with other pro-inflammatory signaling pathways in the pathogenesis of periodontitis needs to be investigated further. Identifying necroptosis inhibitors may lead to the development of new immunotherapies for periodontitis.
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