BMB Reports – Manuscript Submission

Manuscript Draft

Manuscript Number: BMB–18–126

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Article Type: Article

Keywords: Periodontitis; bone loss; purinergic receptor; osteoclast; inflammation

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Running Title: Role of P2X5 on periodontal bone loss

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ABSTRACT

Purinergic receptor signaling is increasingly recognized as an important regulator of inflammation. The P2X family purinergic receptors P2X5 and P2X7 have both been implicated in bone biology, and it has been suggested recently that P2X5 may be a significant

regulator of inflammatory bone loss. However, a role for P2X5 in periodontitis is unknown. The present study aimed to evaluate the functional role of P2X5 in ligature-induced periodontitis in mice. Five days after placement of ligature, analysis of alveolar bone revealed decreased bone loss in $P2rx5^{-/-}$ mice compared to $P2rx7^{-/-}$ and WT control mice. Gene expression analysis of the gingival tissue of ligated mice showed that *IL1b, IL6, IL17a* and *Tnfsf11* expression levels were significantly reduced in $P2rx5^{-/-}$ compared to WT mice. These results suggest the P2X5 receptor may regulate bone loss related to periodontitis and it may thus be a novel therapeutic target in this oral disease.

INTRODUCTION

Periodontitis is a common chronic inflammatory disease that affects the periodontium surrounding and supporting the teeth and represents the most prevalent form of bone pathology in humans. Specifically, almost 50% of adults suffer from some form of periodontitis with approximately 10% of the population being affected by generalized severe periodontitis (1). This disease is initiated by dysbiotic microbial communities that colonize subgingival tooth surfaces and cause gingival inflammation that leads to destruction of periodontal connective tissue and bone (2). The gram-negative bacterium *Porphyromonas gingivalis* is a major factor in the pathogenesis of periodontal disease where it functions as a keystone pathogen (3). The LPS of this pathogen is an important virulence factor and has been implicated in both periodontal disease pathogenesis and associated systemic conditions (4). The immunopathologic lesions in periodontitis are characterized by breakdown of the collagen fibers of the periodontal ligament and the loss of gingival tissue and alveolar bone, which is mediated locally by host-produced inflammatory factors, such as interleukin-1β (IL-

1β), tumor necrosis factor (TNF), IL-6, IL-17 and receptor activator of nuclear factor kappa-B ligand (RANKL). Expression of these factors occurs in response to the dysbiotic microbiota and associated molecular components, including LPS. It is known that cytokines, chemokines, arachidonic acid metabolites, and proteolytic enzymes are collectively pivotal in promoting periodontal inflammation, and in periodontitis are elevated in both the gingival crevicular fluid (5-7) and the gingival tissue (8). Several studies suggest that pro-inflammatory cytokines, including IL-1, IL-4, IL-17, and TNF-α, that are induced during T cell-mediated immune responses, play crucial roles in regulating RANKL expression on osteoblasts, which is associated with inflammation that can affect bone metabolism (9).

The purinergic signaling system has a wide range of physiological and pathological roles (10, 11), including inflammation and bone remodeling, and transmits signals from extracellular nucleotides via cell surface P2 purinergic receptors, which consist of two sub-families, ionotropic receptors (P2X) and metabotropic receptors (P2Y), that are expressed on various cell types (12). Functional expression of multiple P2X and P2Y receptor subtypes has been demonstrated on both osteoblasts and osteoclasts (13, 14). Activation of these receptors by endogenous ligands regulates various cellular functions, including proliferation and apoptosis, that are crucial role for bone remodeling in bone microenvironments (15). In addition, purinergic receptors are involved in the activation of the inflammasome, which is responsible for post-translational processing of IL-1β and IL-18 cytokines to mature forms.

Previous work has reported roles for P2X7 in both osteoblast and osteoclast physiology. However, *P2rx7*-deficient mice and precursor cells maintain capacity to form multinucleated osteoclasts *in vivo* and *in vitro* (16, 17), respectively, suggesting the need for additional investigation into the role of purinergic signaling in bone. Recently, a study from our group

showed that P2X5 is required for optimal inflammasome-induced IL-1β production by osteoclasts, and for osteoclast maturation and hyper-multinucleation (18). Interestingly, inflammasomes have been reported to enhance osteoclast differentiation and bone resorption (19). Inflammation and bone loss are tightly connected with periodontal disease. However, although the actions of purinergic receptors during inflammation are appreciated, nothing is currently known about how P2X5 functions in the context of periodontal disease, or whether P2X5 exerts specific function that is independent of previously reported functions of P2X7 (20). In this study, we used a ligature-induced experimental periodontitis model and found that P2X5 deficiency significantly decreased ligature-induced periodontal bone loss. P2X5 deficiency was also found to inhibit local osteoclast differentiation and activation. These findings suggest that P2X5 (and/or its associated signaling components) may be worth investigating as potential therapeutic targets in the context of periodontal disease and other bone loss disorders.

RESULTS

P2X5-dependent induction of IL-1β and osteoclast multinucleation by P. gingivalis LPS

We recently reported that in a model of inflammatory bone loss, in which LPS from *Escheric hia coli* O111:B4 is injected above the calvarial bone, *P2rx5⁻ᐟ⁻* mice exhibit comparatively low osteoclast numbers and localized bone resorption (18). However, whether P2X5 plays a similar role in the setting of periodontitis is uncertain and was investigated in this study. To test whether *P. gingivalis* LPS-mediated signaling is similarly dependent on P2X5, we cultured BMMs from control and *P2rx5⁻ᐟ⁻* mice, as well as from mice deficient in P2X7, the

P2X member best characterized as an inflammatory activator (21), for two days with M-CSF and RANKL, then cultured for an additional day in the presence or absence of 100 ng/mL *P. gingivalis* LPS. ELISA of culture supernatants revealed defective LPS-induced IL-1β secretion by both *P2rx5$^{-/-}$* and *P2rx7$^{-/-}$* cells compared to control cells (Figure 1A). *P. gingivalis* LPS treatment did not affect osteoclast differentiation as measured by TRAP staining (Figure 1B, upper panels) or numbers of osteoclasts with greater than three nuclei (Figure 1B, lower left plot). In contrast, *P. gingivalis* LPS treatment induced osteoclast hyper-multinucleation, a marker of inflammation-related osteoclast formation defined by the number of cells of greater than 100 μm diameter; this activity was observed in control or *P2rx7$^{-/-}$* cells but was specifically unaffected in *P2rx5$^{-/-}$* cells (Figure 1B, lower right plot). Together these results suggest a potential role for P2X5 in inflammatory bone loss conditions associated with *P. gingivalis*.

P2X5 deficiency decreases alveolar bone loss in ligature-induced periodontitis

To investigate the role of P2X5 in periodontal disease, ligature-induced periodontitis was performed using littermate WT and *P2rx5$^{-/-}$* mice. *P2rx7$^{-/-}$* mice were also tested to determine whether potential effects observed in *P2rx5$^{-/-}$* mice were specific to disruption of a particular P2X receptor, or to P2X signaling more generally. Alveolar bone loss was measured using CEJ-ABC distance. Significant differences in alveolar bone loss between unligated control and corresponding ligated teeth 5 days after surgery were observed for WT and *P2rx7$^{-/-}$* groups, showing that the ligatures around the teeth were able to promote bone loss (Figure 2B). However, alveolar bone loss decreased by 44% in *P2rx5$^{-/-}$* ($P < 0.001$) mice compared with WT mice after ligation (Figure 2A). These data suggest that P2X5 receptor, but not P2X7 receptor, regulate alveolar bone loss in the ligature-induced periodontitis model.

Decreased cytokine expression in P2X5-deficient gingival tissues post-ligation

The matrix metalloproteinases *Mmp2* and *Mmp9* are reported biomarkers for periodontitis (22). We examined *Mmp2* and *Mmp9* expression in the context of our model, and found that while expression of Mmp2 was not significantly induced during periodontitis progression, Mmp9 expression in ligated gingival tissue was lower in *P2rx5*^{-/-} samples compared to those from WT controls (Figure 3A). The elevation of pro-inflammatory cytokines, such as *IL1b, IL6,* and *IL17*, in periodontitis has been found to stimulate alveolar bone loss (23, 24). To examine whether the production of pro-inflammatory cytokines was decreased in *P2rx5*^{-/-} mice, we performed qPCR in gingival tissues of ligature-induced periodontitis from WT and *P2rx5*^{-/-} mice. We observed that induction of *IL1b, IL6, IL17a* and *Tnfsf11* was substantially higher in ligated WT gingival tissue than in ligated *P2rx5*^{-/-} tissue ($P < 0.05$). In contrast, there were no significant differences in *IL23a, IL10* and *Tnfsf11b* expression in the unligated versus ligated gingiva in WT and *P2rx5*^{-/-} mice (Figure 3B). These data suggest that in addition to regulating osteoclast function, the P2X5 receptor also regulates the host inflammatory response in the setting of experimental periodontitis.

DISCUSSION

In this study, we examined the role of P2X5 in inflammatory events and bone loss associated with periodontal disease. We used a well-established experimental model of ligature-induced periodontitis in mice, wherein ligature placement leads to rapid accumulation of bacteria in the adjacent dentogingival areas that allows for intense host-bacterial interactions. These

interactions cause inflammation that compromises connective and bone tissue integrity, which is evident by day 5 of periodontal disease induction (25). These processes give rise to inflammatory cell influx and destruction of the alveolar bone and other periodontal connective tissues, leading to pathologic destruction of the periodontal tissues and those similarly observed in human periodontitis.

Expression of P2X purinergic receptor family members expression is widespread, and activation by extracellular ATP has variously immunomodulatory effects (21). P2X7 receptor, in particular, has been characterized as an upstream activator of inflammasomes, where, for example, potassium efflux through the P2X7 ion channel triggers the activation of NALP3 inflammasome, resulting in activation of caspase 1 and subsequent cleavage of pro-IL-1β into releasable mature IL-1β (26). *P2rx7*-deficient mice were generated by two independent groups (16, 17) and their bone phenotypes analyzed with somewhat conflicting findings. In one case, *P2rx7* deficiency showed a reduction in total and cortical bone content in the femur, reduced periosteal bone formation, increased trabecular bone resorption in the tibia and a reduced sensitivity to mechanical loading (17). The other model of *P2rx7* deficiency only showed that cortical thickness was increased compared to WT controls (16). In this study, we used the first *P2rx7*-deficient model to examine potential roles in periodontal disease, but found that *P2rx7* deficiency exhibited no significant differences in periodontal bone loss compared to WT controls (Figure 2B). By contrast, it was observed that 65% of periodontitis-associated bone loss was ameliorated in *P2rx5*-deficient mice (Figure 2A).

Excessive production of MMPs leads to accelerated periodontal matrix degradation, and we observed Mmp9 levels are lower in $P2rx5^{-/-}$ mice compared with WT, while in $P2rx7^{-/-}$ mice levels were similar to WT (data not shown). Interestingly, we did not detect any difference in

Mmp2 expression during periodontitis progression. Mmp2 is secreted by gingival fibroblasts and/or osteoblasts and Mmp9 is mainly secreted by polymorphonuclear leukocytes including osteoclasts. For the qPCR analysis, we peeled out soft tissues which contained very low osteoblasts cells from bone surface.

Under inflammatory conditions, the response of the host is primarily mediated by innate immune system activation, causing inflammatory cells to migrate, proliferate, differentiate, or produce diverse inflammatory mediators (27). Cytokines such as TNF, IL-1, and IL-17 promote substantially the pathological bone loss that frequently occurs with periodontal disease (23, 24). Moreover, IL-1 stimulates osteoclast differentiation and bone resorption, largely through up-regulation of the RANK and RANKL, whereas TNF stimulates osteoclast differentiation and activation through RANKL-dependent or independent mechanisms. To investigate the role of P2X5 in periodontitis we used the murine ligature-induced periodontitis model. This is an established model of experimental periodontitis, where placement of a silk suture around molar teeth leads to local accumulation of bacteria and rapid inflammation in surrounding gingival tissues resulting in destruction of the underlying alveolar bone (25). Using this model, we found that the expression of pro-inflammatory cytokines (including *IL1b, IL6* and *IL17a*) in the gingival tissue was inhibited by P2X5 deficiency. These cytokines play critical roles in the development of the periodontitis, which is initiated by microbial dysbiosis (2). IL-17 is important for the recruitment of neutrophils, which are causative in periodontitis as they are responsible for a substantial portion of inflammatory tissue destruction and their numbers correlate positively with the severity of the disease (28, 29). However, a conclusive role of Th17 cells, a major source of IL-17, is uncertain. In this context, a recent study showed that there is significantly increased number of Th17 cells in the gingival tissue of older mice (24 weeks) as compared to 8 weeks old mice (30). Because 24-week-old mice exhibit more bone loss than 8-week-old mice, it appears that

the presence of Th17 cells correlates with increased bone loss. *In vitro* studies show that IL-17 stimulates osteoclast differentiation most likely indirectly through induction of RANKL (31). Moreover, IL-17 pathways have been also shown to have a potentially critical role in the pathogenesis of rheumatoid arthritis, notably in the regulation of bone devastation (31, 32). Our findings that both IL-17 and RANKL were significantly inhibited by P2X5 deficiency suggests that the expression of these cytokines might be associated with P2X5 signaling pathways in periodontal disease progression.

Osteoclasts differentiate from hematopoietic precursors in the monocyte-macrophage lineage at various stage including proliferation, migration, fusion, and activation. Osteoclasts are specialized multinucleated cells involved in bone resorption and increased osteoclast differentiation functionally leads to devastating bone loss-related diseases, such as osteoporosis, periodontitis, rheumatoid arthritis, osteosarcoma, and cancer metastasis to the bone. Recently, we have shown that the purinergic receptor P2X5 plays a pivotal role in regulating osteoclast maturation and hyper-multinucleation (18). Furthermore, *P2rx5*-deficient mice are protected from LPS-induced inflammatory bone loss in an *in vivo* model that targets calvarial bone (18). IL-1β is involved in various cellular and tissue functions including osteoclast differentiation (33), and is regulated by purinergic signaling through inflammasome-mediated conversion of pro-IL-1β into active IL-1β (34). Under physiological conditions, osteoclast differentiation is dependent on RANKL stimulation, but IL-1β can induce RANKL-independent osteoclast differentiation (35). However, it is uncertain whether the P2X5 specifically regulates production of IL-1β in the context of periodontal disease.

Taken together, although a full understanding of how P2X5 signaling contributes to inflammatory bone loss *in vivo* remains unclear, we provide evidence that this pathway drives

experimental periodontitis via promotion of inflammation and direct regulation of osteoclast maturation, thereby suggesting a potential candidate for therapeutic targeting in periodontal disease.

MATERIALS AND METHODS

Mice

Experiments were performed on 9 week-old female $P2rx5^{-/-}$ (18) and $P2rx7^{-/-}$ (The Jackson Laboratory, stock #005576) mice (both on the C57BL/6 background). The experimental protocol for surgical procedures and animal treatments was approved by Institutional Animal Care and Use Committee of the University of Pennsylvania (Animal protocols #805528 and #804178).

ELISA assay

The ELISA for IL-1β was performed following the manufacturer's instructions (BD Biosciences). Briefly, collected culture media were filtered through a 0.45 μm syringe filter, added to a well coated with anti-IL-1β capture antibody, and incubated overnight at 4°C. Samples from each well were then removed and the wells washed, and 100 μl of biotin-antibody working solution was then added to each well for 1 h. The mixture was removed and the wells washed before HRP-avidin working solution was added to each well for 1 h incubation. After washing, TMB substrate was added and incubated for 30 min, and the reaction was stopped by adding 50 μl of stop solution (1M H_3PO_4) to each well. The optical density of each well was determined using a microplate reader (Molecular Devices Vmax,

Menlo Park, CA, USA) set to 450 nm.

Osteoclast differentiation and tartrate-resistant acid phosphatase (TRAP) staining

Analysis of osteoclast differentiation was performed as previously reported (36, 37). Briefly, BMMs were generated from cultures of bone marrow cells and re-plated at 2×10^4 per well in 96-well cell culture plates. Cells were cultured for 3 days with M-CSF (60 ng/mL) and RANKL (150 ng/mL). Cells were then fixed with 3.7% formalin for 30 min and stained using the Acid, Phosphatase, Leukocyte (tartrate-resistant acid phosphatase) Kit (387A-1KT, Sigma). TRAP-positive multi-nuclear cells (MNCs) were counted under light microscopy (Nikon).

TRAP activity assay

TRAP assays were carried out as previously described (38). Briefly, osteoclasts in a 96 well plate were fixed in 3.7% (v/v) formalin for 10 min, washed with DDW, then incubated with 150 μl of TRAP buffer (120 mM Na-acetate and 52 mM Na-tartrate, pH 5.2) containing 1 mg/mL *p*-nitrophenylphosphate (Sigma) for 1 h at 37°C. The reaction mixtures were then transferred to a new plate containing 50 μl of 1N NaOH. Absorbance was measured at 405 nm using a microplate reader (Molecular Devices Vmax, Menlo Park, CA, USA).

Ligature-induced periodontal disease

P2rx5$^{-/-}$ and *P2rx7*$^{-/-}$ mice and their littermate controls (*P2rx5*$^{+/+}$ or *P2rx7*$^{+/+}$) were co-housed for at least for 4 weeks prior to experiments. Anesthesia was induced in mice by intraperitoneal injection of ketamine and xylazine. A 5-0 silk ligature was tied around the maxillary left second molar and the mice were euthanized five days after placement of the ligatures. The contralateral molar tooth in each mouse was left unligated as a baseline control

for bone loss measurements. Periodontal bone loss was assessed morphometrically in defleshed and stained maxillae. Images were taken using a dissecting microscope (40x) fitted with a video image measurement system (Nikon Instruments). Specifically, the distance between cementoenamel junction (CEJ) and alveolar bone crest (ABC) was measured on six predetermined points on the ligated second molar and the affected adjacent regions (25). Bone loss was calculated by subtracting the 6-site total CEJ-ABC distance for the ligated side of each mouse from the 6-site total CEJ-ABC distance of the contralateral unligated side. Negative values (in mm) indicated bone loss relative to the baseline (unligated control).

Real time-PCR (qPCR)

Isolation of total RNA, and cDNA synthesis, were performed from cells with the use of Trizol reagent (Invitrogen) or from gingival tissues with the use of the RNeasy Mini Kit (QIAGEN), and RNA was reversed transcribed by using random hexamer primers and Superscript III reverse transcriptase (Invitrogen). In brief, quantitative qPCR was performed as described previously (39). The synthesized cDNA was analyzed by qPCR using an ABI PRISM 7300 or 7500 real time PCR instrument (Applied Biosystems) and specific Taqman™ probes as follows: Mmp2 (Mm00439498_m1), Mmp9 (Mm00442991_m1), IL1b (Mm00434228_m1), IL17a (Mm00439618_m1), IL23a (Mm00518984_m1), IL6 (Mm00446190_m1), Tnfsf11 (Mm00441906_m1), Tnfsf11b (Mm00435454_m1), IL10 (Mm01288386_m1), P2rx5 (Mm00473677_m1), and Gapdh (Mm99999915_g1). Three technical replicates per sample and gene were performed. Amplification parameters were 5 min of denaturation and activation at 95ºC and 50 cycles of 30 s at 95ºC and 1 min at 60ºC. The C_T method of relative quantification was used to determine the fold change in expression.

Statistics

Statistical analysis was performed using GraphPad Prism Ver. 6.0e (GraphPad, San Diego, CA). For determining statistical significance, a two-tailed unpaired t-test (for comparing two groups) or one-way ANOVA with a Tukey's multiple comparisons test (for comparing three or more groups) was used. Values of $P < 0.05$ were considered significant.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. P2X5-dependent induction of IL-1β and osteoclast multinucleation by *P. gingivalis* LPS. BMMs were cultured for 2 days in the presence of M-CSF and RANKL, then cultured for 1 day with or without *P. gingivalis* LPS (100 ng/mL). A. IL-1β secretion into the culture medium was analyzed by ELISA. B. Cells were fixed and stained for TRAP (upper panels). TRAP+ MNCs were counted both by the presence of more than 3 nuclei (lower left plot) and cell size larger (lower right plot) than 100 μm in diameter. Scale bar represents 200 μm. Data are means ± SD. Data represent three independent experiments, each performed in three replicas. *$P < 0.05$. ***$P < 0.001$. NS, not significant.

Figure 2. Induction of periodontal bone loss in *P2rx5$^{-/-}$* and *P2rx7$^{-/-}$* mice. Representative images of maxillae (upper jaws) illustrating the morphometric findings of *P2rx5$^{-/-}$* (A) and

P2rx7⁻ᐟ⁻ (B). Determination of bone changes in the jaws was calculated by measuring the CEJ-ABC distance for each mouse and transforming the data to directly indicate bone loss (lower panel on each). Data are means ± SD. ==Data pooled from two independent experiments with *n* = 6-8 mice per group.== **P < 0.01. ***P < 0.001. ****P < 0.0001. NS, Not significant.

Figure 3. ==Matrix metalloproteinases (Mmp) and== cytokine mRNA expression levels at the ligated vs. unligated gingival tissues between WT and *P2rx5⁻ᐟ⁻* mice. ==(A) Expression of *Mmp2* and *Mmp9* in gingival tissues.== (B) Expression of *IL1b, IL6, IL23a, IL17a, Tnfsf11, Tnfsf11b, IL10* and *P2rx5* in gingival tissues, as determined by qPCR. Data are normalized to Gapdh and are presented as fold change in the transcript levels in ligated sites relative to those of unligated sites, set as 1. Data are means ± SD. ==Data pooled from two independent experiments with *n* = 6-8 replicates per group.== *P < 0.05. ND., Not determined. NS., Not significant.

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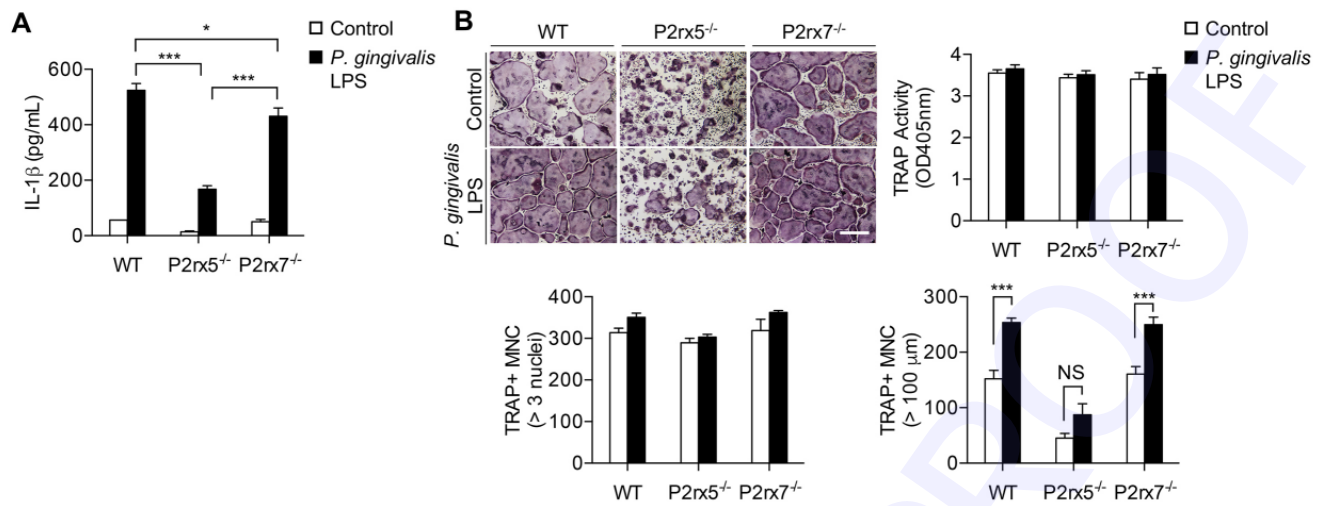
Fig. 1

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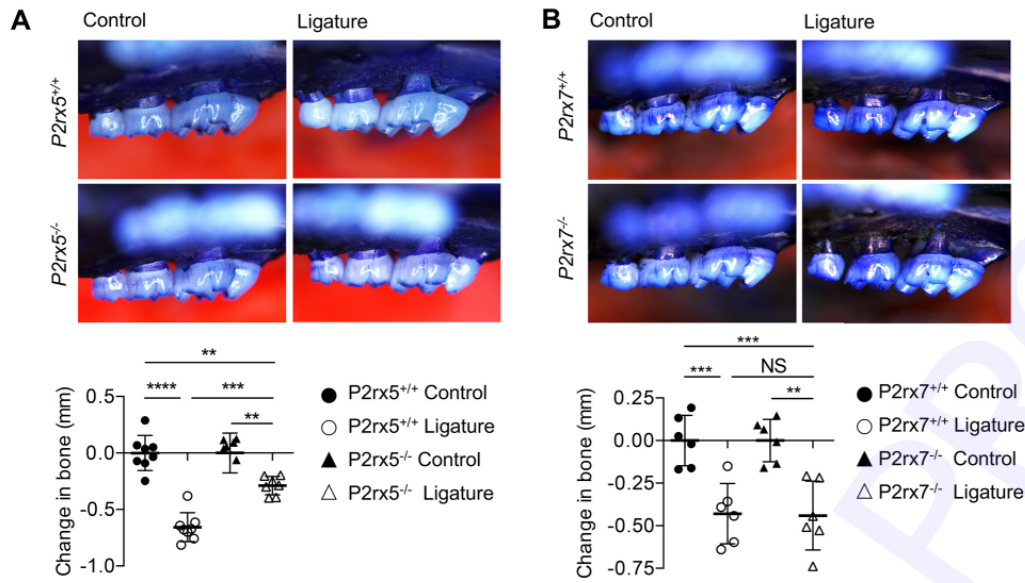
Fig. 2

Fig. 2. Induction of periodontal bone loss in P2rx5-/- and P2rx7-/- mice. Representative images of maxillae (upper jaws) illustrating the morphometric findings of P2rx5-/- (A) and P2rx7-/- (B). Determination of bone changes in the jaws was calculated by measuring the CEJ-ABC distance for each mouse and transforming the data to directly indicate bone loss (lower panel on each). Data are means
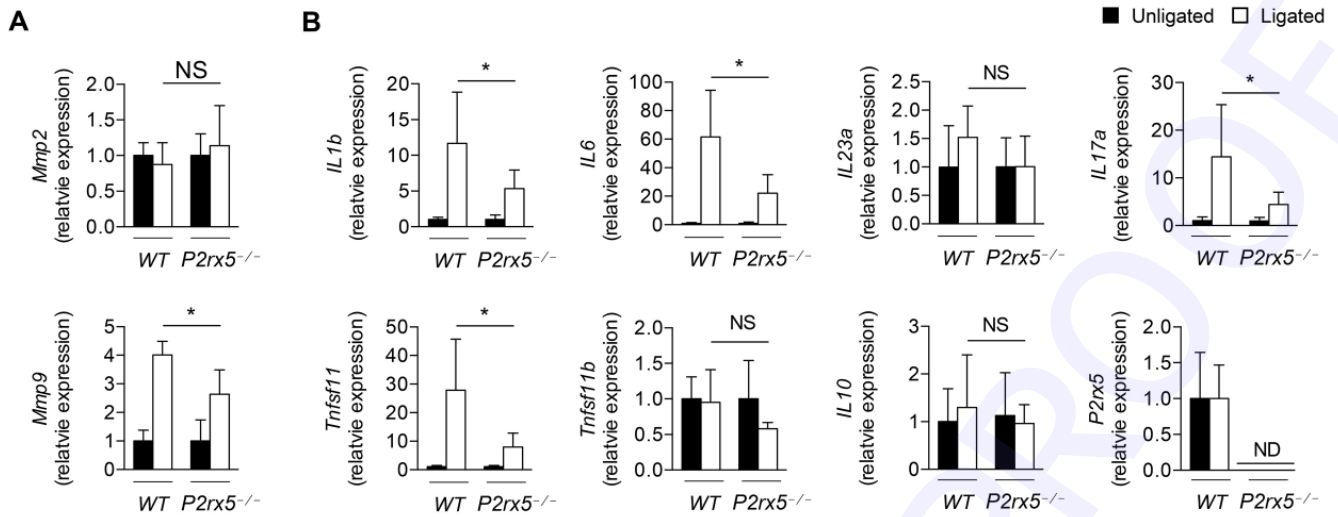
Fig.3

Fig. 3. Matrix metalloproteinases (Mmp) and cytokine mRNA expression levels at the ligated vs. unligated gingival tissues between WT and P2rx5-/- mice. (A) Expression of Mmp2 and Mmp9 in gingival tissues. (B) Expression of IL1b, IL6, IL23a, IL17a, Tnfsf11, Tnfsf11b, IL10 and P2rx5 in gingival tissues, as determined by qPCR. Data are normalized to Gapdh and are presented as fold change in the transcript levels in ligated sites relative to those of unligated sites, set as 1. Data are means