Reduced expression of lipopolysaccharide-induced CXC chemokine in Porphyromonas gingivalis-induced experimental periodontitis in matrix metalloproteinase-8 null mice


Background and Objective: Matrix metalloproteinase-8 (MMP-8) is a central mediator in chronic periodontitis. Recently developed MMP-8-deficient mice show an impaired polymorphonuclear neutrophil response and more severe alveolar bone loss in Porphyromonas gingivalis-induced experimental periodontitis. The main mediators involved in neutrophil and monocyte/macrophage recruitment and in bone loss include lipopolysaccharide-induced CXC chemokine (LIX/CXCL5), stromal-derived factor-1/CXC chemokine ligand 12 (SDF1/CXCL12) and RANKL. Therefore, the aim of this study was to characterize the expression of LIX/CXCL5, SDF1/CXCL12 and RANKL in Porphyromonas gingivalis-induced experimental periodontitis in MMP-8−/− (knockout) and wild-type mice.

Material and methods: MMP-8 null and WT P. gingivalis-infected and uninfected mice were included. Histopathological changes were assessed and LIX/CXCL5, SDF1/CXCL12 and RANKL were immunodetected and quantified.

Results: Typical histopathological features of chronic periodontitis were seen in P. gingivalis-infected groups. LIX/CXCL5 expression was restricted to the gingival papilla in all four groups. Significantly lower expression of LIX/CXCL5 was seen in the knockout group compared with the wild-type infected group (p < 0.05). SDF1/CXCL12 and RANKL expression was mainly localized to the alveolar crest, including inflammatory leukocytes, vascular endothelium, osteoblasts and osteoclasts. Significant increases of SDF1/CXCL12 and RANKL were
seen in both knockout and wild-type *P. gingivalis*-infected groups compared with uninfected groups (*p < 0.05*).

**Conclusion:** RANKL and SDF1/CXCL12 are up-regulated in *P. gingivalis*-induced periodontitis and they appear to be associated with the pathogenesis of the disease. MMP-8 is associated with a reduced expression of LIX/CXCL5 in the *P. gingivalis*-induced experimental periodontitis model.

Periodontitis develops from a heterogeneous etiologic interaction between bacterial infection and the host response (1). Periodontal disease is initiated by a complex bacterial biofilm localized in the subgingival environment, where *Porphyromonas gingivalis* is regarded as one of the major causative pathogens (2). Infection triggers a host immune-inflammatory response, resulting in periodontal tissue destruction (1). Release of inflammatory mediators, such as chemokines, cytokines and matrix metalloproteinases (MMPs), may induce further vascular and cellular changes, such as the formation of an inflammatory infiltrate, destruction of alveolar bone and apical migration of the junctional epithelium (1,3).

MMP-8 is the major collagenolytic MMP, and elevated levels of this enzyme have been associated with severe periodontal inflammation and disease (1,4–8). MMP-8 degrades the main extracellular matrix components from the periodontium, and also regulates the immune response through the bioavailability and biological activity of cytokines by the proteolytic processing of nonmatrix bioactive molecules (9–11). Furthermore, recent reports have demonstrated changes in cytokine protein levels that are associated with the activity and levels of MMPs (12,13).

To date, the biological functions of MMP-8 are uncertain (13), although an impaired inflammatory response was found in MMP-8-deficient mice models, characterized by reduced neutrophil infiltration and reduced levels of the cytokine transforming growth factor-β1 (TGF-β1) (10,13–15). The recent development of a *P. gingivalis*-induced model of periodontitis showed severe disease with unexpectedly extensive alveolar bone resorption in MMP-8-deficient mice. Altogether these data support a role for MMP-8 in inflammatory response and bone resorption through cytokine regulation (16).

Neutrophils play a pivotal role as a first line of defense against pathogens in periodontal homeostasis, whilst alterations in neutrophil function are associated with severe periodontal disease (17). Neutrophil recruitment follows the gradient of specific chemokines secreted in response to microbial products or proinflammatory cytokines. Mouse lipopolysaccharide-induced CXC chemokine (LIX/ CXCL5) is the most abundant and potent chemoattractant for neutrophils *in vitro* and *in vivo*, representing the sole murine homolog of two closely related human chemokines, CXCL5 and CXC chemokine ligand 6 (CXCL6) (17–19). MMP-8-deficient mice have shown decreased levels of polymorphonuclear neutrophil (PMN) infiltration resulting from reduced LIX/CXCL5 mobilization from the extracellular matrix (10). Inflammation and bone resorption during periodontitis have been widely associated with high RANKL levels and, recently, high stromal-derived factor-1/CXC chemokine ligand 12 (SDF-1/CXCL12) levels (20–22). SDF-1/CXCL12 induces monocyte/macrophage recruitment via activation of the CXC chemokine 4 receptor (CXCR4) and, together with RANKL, SDF-1/ CXCL12 promotes early and late stages of osteoclastogenesis (21,23).

Based on the previous findings, we hypothesize that increased disease severity and alveolar bone loss in the *P. gingivalis*-induced model of periodontitis results from reduced levels and/or bioavailability of the key inflammatory mediators LIX/CXCL5, SDF1/CXCL12 and RANKL. In the current study, we aimed to characterize the expression patterns and levels of these inflammatory chemokines and cytokines in *Porphyromonas gingivalis*-induced experimental periodontitis in MMP-8-deficient and wild-type (WT) control mice.

**Material and methods**

**Animals**

Animals and tissue specimens included and analyzed in the current study correspond to those reported previously by Kuula et al. (16). Briefly, experimental groups comprised 14-wk-old male mice bred and maintained in the experimental animal facilities of the University of Oulu, Oulu, Finland. MMP-8<sup>+/−</sup> [knockout (KO)] mice of a mixed C57BL/6J/129 background (15) and WT littermates, resulting from intercrossing of heterozygous mice, were included as controls (14,15,24). The MMP-8 KO mice were kindly provided by Carlos López-Otin (Department of Biochemistry and Molecular Biology, University Institute of Oncology, Universidad de Oviedo, Oviedo, Spain). All mice were maintained in a barrier facility (24), and the experiments were conducted in accordance with the guidelines of the Animal Experimentation Committee of University of Oulu, Oulu Finland.

**Induction of experimental periodontitis**

The experimental groups were assigned as follows: WT-infected (experimental, *n* = 10); WT uninfected (control, *n* = 8); MMP-8 KO infected (experimental, *n* = 12); and MMP-8 KO uninfected (control, *n* = 10) (total *n* = 40). Statistical power analysis, based on a pilot study, was performed to determine an appropriate sample size. Experimental periodontitis induction is described in Kuula et al. (16).
Briefly, the mice received 20 mg of kanamycin and 20 mg of ampicillin in 1 mL of sterile water, twice daily for 3 d, to eliminate the native flora from the oral cavity. The antibiotics were allowed to clear from the system for 4 d and then the mice were inoculated orally with *P. gingivalis* (strain ATCC33277; American Type Culture Collection, Manassas, VA, USA). The bacteria used for oral inoculation were prepared as follows: After expansion culture, the bacteria were suspended in sterile carboxymethyl cellulose (3%) at a concentration of ~2 × 10^8 CFU/mL (25,26). Then, 0.1–0.2 mL of the carboxymethyl cellulose suspension, containing viable *P. gingivalis*, was swabbed intra-orally, twice a day, for 3 d. The control mice receiving saline served as negative controls. Thirty days after the last inoculation the mice were killed by cervical dislocation. The skulls were dissected, hemisected, fixed in 10% formalin, decalcified in 12.5% EDTA, embedded in paraffin and processed for routine histopathology. The sections were stained with hematoxylin and eosin (H&E) and immunohistochemically analyzed.

**Immunohistochemistry**

Paraaffin-embedded specimens were cut, deparaaffinized, pretreated with 0.4% pepsin, and the endogenous peroxidase activity was quenched by incubation in H_2O_2/methanol. Samples were blocked with normal serum containing 2% bovine serum albumin and incubated with primary antibodies (Vector Laboratories, Burlingame, CA, USA) and 3-amino-9-ethylcarbazole as a chromogen, counterstained with Mayer's hematoxylin (Merck KGaA, Darmstadt, Germany) and mounted in Dako Glycergel (Dako Corp., Carpinteria, CA, USA). Randomly selected images were acquired using an Olympus BX61 microscope fitted with an Olympus DP50 camera and UPlanFl 40×0.75 NA and 20×0.50 NA objectives. The number of immunoreactive cells and total stromal and inflammatory cells, and the size of the areas containing positively staining gingival epithelial cells and all gingival epithelial cells were analyzed using a macro written for the public domain ImagemJ software. Image preprocessing included median and watershed filtering for total cells and maximum entropy thresholding for immunoreactive cells (http://rsbweb.nih.gov/ij). Histopathological analysis was performed blindly by a single evaluator. The results were expressed as the mean ratio of positive/total cells, or the area of immunoreactive gingival epithelium at measurable periodontal sites.

**Statistical analysis**

Statistical significances were calculated using the one-way analysis of variance with Bonferroni's post hoc test for intergroup analyses with parametric data distribution; the Mann–Whitney test for two-group comparisons with a nonparametric distribution and the chi-square test for dichotomic data analyses. Spearman's correlation was used to identify associations among chemokine and cytokine levels. A p-value of < 0.05 was considered to be statistically significant. Statistical analyses were performed using Stata V10 software (StataCorp, College Station, TX, USA).

**Results**

Typical histopathological changes associated with chronic periodontitis were observed in hematoxylin and eosin-stained sections of periodontal tissues from both WT and MMP-8 KO *P. gingivalis*-infected groups, but not in noninfected mice. Histopathology analyses showed extensive epithelial proliferation and apical migration, reduced alveolar crest bone height and infiltration of inflammatory cells (Fig. 1A). Expression of the chemokines LIX/CXCL5 and SDF1/CXCL12, and of the cytokine RANKL, was quantified in all periodontal sites and expressed as the ratio of immunoreactive/total cells, or the area of immunoreactive gingival epithelium/total area at the periodontal sites.

Expression of LIX/CXCL5 in periodontal tissues was detected in all four study groups: *P. gingivalis*-infected WT; *P. gingivalis*-infected MMP-8 KO; uninfected WT; and uninfected MMP-8 KO (Table 1, Fig. 1B). The expression of LIX/CXCL5 was restricted to gingival papilla, and it was extensively immunolocalized to the gingival epithelium and subepithelial inflammatory cells in *P. gingivalis*-infected groups (Fig. 2A). Quantification of LIX/CXCL5 expression in the gingival papilla resulted in significantly higher levels of LIX/CXCL5 in the *P. gingivalis*-infected WT group, compared with both infected and uninfected MMP-8 KO groups (Fig. 2B).

SDF1/CXCL12 and RANKL expression patterns (Fig. 1B) were more diffuse and both were mainly expressed in both *P. gingivalis*-infected groups, in bone marrow stromal cells (Figs 3A and 4A), periodontal tissues (Figs 3B and 4B) and also in dental pulp cells (Fig. 1B), and their patterns of tissue distribution were similar between KO-infected group and WT-infected group. The levels of SDF1/CXCL12 and RANKL in periodontal tissues from uninfected mice were undetectable in most of the cases (Figs 3B and 4B).

In infected mice, SDF1/CXCL12 was expressed weakly in the gingival epithelium and strongly in the periodontal ligament and inflammatory leukocytes close to areas of alveolar bone resorption, and expressed variably in osteoblasts (Fig. 3A). The strongest expression of SDF1/CXCL12 in periodontal tissues was localized to vascular channels surrounding the alveolar crest (Fig. 3B). RANKL was weakly expressed in the gingival epithelium and strongly expressed in osteoclasts and epithelial rests of Malassez (Fig. 4A); however, RANKL was expressed most strongly in the periodontal ligament and inflammatory cells surrounding the alveolar crest (Fig. 4B).

As shown in Table 1, the detection frequencies of SDF1/CXCL12 and RANKL were higher in *P. gingivalis*-infected mice than in uninfected mice.
No significant differences were found in the expression levels of SDF1/CXCL12 and RANKL between infected WT and MMP-8 KO groups (Figs 3C and 4C, respectively). Moreover, a significant, positive correlation was found between SDF1/CXCL12 and RANKL ($r = 0.40, p = 0.01$). Analysis of LIX/CXCL5, SDF1/CXCL12 and RANKL by localization among different maxillary and mandibular sites did not show differences in their distribution among sites in the study groups ($p > 0.05$).

**Discussion**

Periodontal diseases are characterized by elicitation of chronic inflammatory process by pathogenic bacteria from subgingival plaque, resulting in characteristic soft-tissue destruction and alveolar bone resorption. A wide variety of cytokines, chemokines and their receptors are synthesized by resident and inflammatory cells, either potentiating or regulating the immune response in periodontal tissues (27). MMP-8 is not only the major collagenolytic MMP associated with severity of periodontal disease (1,5,9,28–31), but is also a central mediator in chronic infection-induced inflammatory conditions and can exert immune-regulatory properties, in addition to the classical surrogate tissue-destructive properties (16).

We investigated the role of MMP-8 on chemokine and cytokine expression in *P. gingivalis*-induced periodontitis in a mouse model deficient in MMP-8. Our current results indicate a potential inflammatory role of MMP-8 during periodontitis through the regulation of LIX/CXCL5. Additionally, *P. gingivalis* infection resulted in increased expression of RANKL and SDF1/CXCL12, irrespective of the MMP-8 genotype, suggesting important roles in disease initiation and/or progression.

Expression of the chemokine LIX/CXCL5 was detected in periodontal tissues from all study groups (*P. gingivalis*-infected and uninfected MMP-8 KO and WT mice) and showed a particularly restricted pattern, being localized to the epithelium of gingival papilla and some subepithelial inflammatory leukocytes. The expression of LIX/CXCL5 in the gingival epithelium could be involved in regulating neutrophil influx to periodontal tissues at the oral interface where they represent the first line of defense against periodontal pathogens (16). Consequently, the epithelial cells may represent important regulators of inflammation in both healthy and diseased gingival tissue. Recently, differential expression of the LIX/CXCL5 human homolog
granulocyte chemotactic protein-2 (GCP-2)/CXCL6 was reported in diseased vs. healthy gingival tissues. Furthermore, GCP-2/CXCL6 was the most strongly up-regulated cytokine among all known chemokines in periodontal disease, correlating positively with clinical parameters and periodontal pathogens (17).

In our current study we found LIX/CXCL5 expression to be significantly reduced in *P. gingivalis*-infected MMP-8 KO mice, compared with WT groups. MMP-8 is the most important collagenase described in periodontitis and it is believed to be produced primarily by PMNs (11,13). Several studies demonstrate a role for MMP-8 in the regulation of PMN trafficking in different models of inflammation, through (i) hydrolysis of the collagen matrix to allow cell migration, (ii) the cleavage of chemokine-binding proteins and the release of LIX/CXCL5 from the extracellular matrix reservoir and (iii) the processing of LIX/CXCL5, potentiating its biological activity. These potential regulatory mechanisms, together or alone, may lead to impaired influx of PMNs to the sites of inflammation in MMP-8-deficient mice (10,11,13,14,32,33). In line with these findings, our group previously reported a trend of reduced expression of PMN markers – myeloperoxidase and neutrophil elastase – that was associated with more severe alveolar bone loss (1,8) in MMP-8 KO mice compared with WT mice in the same *P. gingivalis*-infected groups of mice (16).

Neutrophils are generally considered as protective against periodontitis as a result of their ability to phagocytose and kill oral bacteria (34). Induction of LIX/CXCL5 expression by oral microbes in periodontal disease may serve to initiate the innate immune response and thereby promote the infiltration of bone-protective neutrophils (19). Based on our preliminary results, MMP-8/LIX/CXCL5 interactions could include a regulatory mechanism resulting in increased LIX/CXCL5 expression, either through increasing protein levels in a feed-forward manner or by mediating LIX/CXCL5 release from the extracellular matrix (11–13). Accordingly, changes in cytokine levels in association

**Fig. 2.** Lipopolysaccharide-induced CXC chemokine (LIX/CXCL5) expression in gingival papilla from matrix metalloproteinase-8 (MMP-8) knockout (KO) and wild-type (WT) mice infected or not infected with *Porphyromonas gingivalis*. (A) LIX/CXCL5 immunostaining. CT, connective tissue; GE, gingival epithelium. The arrowhead shows immunostaining of subepithelial inflammatory cells. 1. WT mice infected with *P. gingivalis*; 2. KO mice infected with *P. gingivalis*; 3. WT uninfected mice; 4. KO uninfected mice. (B) LIX/CXCL5 levels in MMP-8 KO and WT mice infected with *P. gingivalis* and controls. WT + Pg, wild-type mice infected with *P. gingivalis* (n = 20); KO + Pg, knockout mice infected with *P. gingivalis* (n = 24); WT − Pg, wild-type uninfected mice (n = 24); KO − Pg, knockout uninfected mice (n = 24). Results are expressed as the mean ratio ± SD of immunoreactive/total area of gingival epithelium. *p < 0.05.
with MMP levels and activity have been recently reported (12,13). Low-to-undetectable LIX/CXCL5 levels were found in liver tissue from MMP-8 KO mice compared with wild-type mice in a tumor necrosis factor-induced lethal hepatitis model. As a possible explanation, the authors suggested that LIX remained associated with the extracellular matrix in the absence of MMP-8, but the possibility that the expression of LIX was increased as a result of the heightened inflammatory response in MMP-8 WT mice could not be excluded (10). Although a semiquantitative approach was used in the present study, the results are in agreement with previous findings. MMP-9 activity was shown to down-regulate TGF-β1 protein levels in breast cancer cells exposed to tamoxifen (12), and reduced levels of TGF-β1 in MMP-8−/− mice vs. WT mice were associated with impaired PMN infiltration and wound healing (13). Consequently, in addition to a more destructive periodontitis phenotype, MMP-8−/− animals could also present a reduced reparative response. In a wound-healing model, the levels of several cytokines, including TGF-β1, tumor necrosis factor-α, interleukin-1β, keratinocyte-derived chemokine (KC) and MIP-2 were determined but only a reduction in the amount of TGF-β1 was found. In mouse, KC, LIX and MIP-2 are principally involved in the chemotaxis of PMN cells, but among them, only LIX seems to be associated with MMP-8 (15). Because cytokines are pleiotropic and redundant, further studies will be required to determine if a reduced amount of LIX results in functional changes.

Although we cannot exclude the possibility that the mixed background of MMP-8−/− mice could contribute to the differences observed in LIX expression, it is highly improbable because WT control mice had a similar genetic background, and all mice were produced from the intercrossing of MMP-8+/- mice. The previous finding, that *P. gingivalis* inoculation resulted in more severe alveolar bone loss in MMP-8 KO mice (16), led us to investigate whether this finding was mediated through regulation of the expression of the osteoclastogenic chemokine SDF1/CXCL12 and the cytokine RANKL. Our results showed significantly increased expression of both mediators in infected mice, which was not related to the MMP-8 genotype, suggesting

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**Fig. 3.** Stromal-derived factor-1/CXC chemokine ligand 12 (SDF1/CXCL12) expression in matrix metalloproteinase-8 (MMP-8) knockout (KO) and wild-type (WT) mice infected or not infected with *Porphyromonas gingivalis*. (A) SDF1/CXCL12 immunostaining. 1. Bone marrow stromal cells; 2. Osteoblasts and the inflammatory infiltrate from a *P. gingivalis*-infected WT mouse. AC, alveolar crest; BM, bone marrow stromal cells; IC, inflammatory cells; OB, osteoblasts. (B) SDF1/CXCL12 immunostaining in periodontal tissues from 1. WT mice infected with *P. gingivalis*; 2. KO mice infected with *P. gingivalis*; 3. WT uninfected mice; 4. KO uninfected mice. AC, alveolar crest; VC, vascular channels. (C) SDF1/CXCL12 levels in periodontal tissues from *P. gingivalis*-infected mice. WT + Pg, WT mice infected with *P. gingivalis* (n = 44); KO + Pg, KO mice infected with *P. gingivalis* (n = 51). Results are expressed as medians (interquartile range) of the ratio of positive/total cells; p = 0.12.
that SDF1/CXCL12 and RANKL may be overexpressed in response to *P. gingivalis* infection and contribute to the pathogenesis of periodontitis.

Whereas RANKL is the crucial mediator required, along with the permissive factor, colony-stimulating factor 1, for the full development of osteoclast precursors into mature, multinucleated bone-resorbing cells, it can also stimulate osteoclast function and survival (21,35). Our results are in agreement with, and further extend, several reports demonstrating increased RANKL levels and an increased RANKL/osteoprotegerin ratio in periodontitis or in association with *P. gingivalis* infection (36–44). SDF1/CXCL12 is essential for hematopoietic cell homing to the bone marrow and participates in the promotion of chemotaxis and in the early development, cell function, bone resorption and survival of human osteoclasts and their monocytic precursors in a manner similar to the early effects of the monocyte-colony-stimulating factor + RANKL system (21). Accordingly, the expression of RANKL and SDF1/CXCL12 in *P. gingivalis*-infected groups was confined to the area surrounding alveolar bone and to osteoclasts, and a positive correlation was found, suggesting a cooperative effect between them. The strongest expression of SDF1/CXCL12 was localized to vascular channels surrounding the alveolar crest and bone marrow stromal cells. Therefore, SDF1/CXCL12 may direct circulating osteoclast precursors and mononuclear...
leukocytes to the alveolar crest, and stimulate the migration of mononuclear cells within the bone marrow to perivascular periodontal tissue to complete their differentiation to osteoclasts via RANKL and cause alveolar bone loss (20,21). In accordance with this, induction of SDF1/CXCL12 in mice has previously been demonstrated in both a lipopolysaccharide-mediated model of periodontitis (27) and in human periodontal inflammation (20).

Overall, we analyzed cytokine expression in a P. gingivalis-induced periodontitis mouse model deficient in MMP-8 and present initial evidence of increased expression of SDF1/CXCL12 and RANKL in infected mice, suggesting that the development and progression of chronic periodontitis observed in our model might be mediated, at least in part, by SDF1/CXCL12 and RANKL. Reduction of LIX/CXCL5 levels in MMP-8 KO P. gingivalis-infected mice compared with WT mice suggests that MMP-8 regulates LIX/CXCL5. Further studies are required to elucidate whether decreased levels of LIX/CXCL5 influence PMN chemotaxis and function and the periodontal disease phenotype.

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