CXCL1, CCL2, and CCL5 modulation by microbial and biomechanical signals in periodontal cells and tissues—in vitro and in vivo studies

Birgit Rath-Deschner1 • Svenja Memmert1,2 • Anna Damanaki3 • Marjan Nokhbehsaim2 • Sigrun Eick4 • Joni A. Cirelli5 • Werner Götz1 • James Deschner3 • Andreas Jäger1 • Andressa V. B. Nogueira3

Received: 24 October 2019 / Accepted: 12 February 2020 / Published online: 2 March 2020 © The Author(s) 2020

Abstract

Objectives This study was established to investigate whether the chemokines CXCL1, CCL2, and CCL5 are produced in periodontal cells and tissues and, if so, whether their levels are regulated by microbial and/or mechanical signals.

Materials and methods The chemokine expression and protein levels in gingival biopsies from patients with and without periodontitis were analyzed by RT-PCR and immunohistochemistry. The chemokines were also analyzed in gingival biopsies from rats subjected to experimental periodontitis and/or orthodontic tooth movement. Additionally, chemokine levels were determined in periodontal fibroblasts exposed to the periodontopathogen Fusobacterium nucleatum and mechanical forces by RT-PCR and ELISA.

Results Higher CXCL1, CCL2, and CCL5 levels were found in human and rat gingiva from sites of periodontitis as compared with periodontally healthy sites. In the rat experimental periodontitis model, the bacteria-induced upregulation of these chemokines was significantly counteracted by orthodontic forces. In vitro, F. nucleatum caused a significant upregulation of all chemokines at 1 day. When the cells were subjected simultaneously to F. nucleatum and mechanical forces, the upregulation of chemokines was significantly inhibited. The transcriptional findings were paralleled at protein level.

Conclusions This study provides original evidence in vitro and in vivo that the chemokines CXCL1, CCL2, and CCL5 are regulated by both microbial and mechanical signals in periodontal cells and tissues. Furthermore, our study revealed that biomechanical forces can counteract the stimulatory actions of F. nucleatum on these chemokines.

Clinical relevance Mechanical loading might aggravate periodontal infection by compromising the recruitment of immunoinflammatory cells.

Keywords Orthodontic tooth movement • Fusobacterium nucleatum • Periodontitis • Gingivitis • Periodontium

Introduction

Periodontitis is a chronic inflammatory disease characterized by the loss of alveolar bone and clinical attachment [1, 2]. If untreated, the disease can finally result in tooth loss and therefore compromised masticatory performance. Although the disease is multifactorial, microorganisms located on the tooth surface and next to the periodontal tissues are a prerequisite for the initiation and development of periodontitis. Among these microorganisms, Fusobacterium nucleatum plays a critical role, because it acts as a bridge bacterium, i.e., it enables other pathogenic microorganisms to colonize the biofilm [3, 4]. These microorganisms in concert with other risk factors of periodontitis induce an inflammatory host response which is mounted to control and finish the microbial attack. The response by the host comprises a lot of professional and
accessory immunoinflammatory cells, which produce a wide array of inflammatory mediators, matrix-degrading enzymes, and osteoclast-activating molecules [2].

Chemokines, i.e., chemotactic cytokines, are of utmost importance in inflammatory processes, as they ensure a sufficient recruitment of immunoinflammatory cells to the site of microbial infection, e.g., gingiva [5]. Therefore, they play a critical role in chronic inflammatory diseases such as atherosclerosis, osteoarthritis, rheumatoid arthritis, obesity, and periodontitis [6–11]. CXCL1, also referred to as GROα, is a strong chemoattractant for neutrophils which form the first line of defense in the gingival sulcus. The neutrophils can eliminate or reduce bacteria through several mechanisms, such as phagocytosis, extracellular killing by secretion of granule, or reduce bacteria through several mechanisms, such as defense in the gingival sulcus. The neutrophils can eliminate or reduce bacteria through several mechanisms, such as phagocytosis, extracellular killing by secretion of granule, and formation of extracellular traps [12, 13]. In contrast to CXCL1, the chemokines CCL2 and CCL5, also known as MCP-1 and RANTES, respectively, mainly facilitate the recruitment of monocytes, macrophages, and T lymphocytes, which are located below the gingival epithelium, where they form the second line of defense [13]. Therefore, regulation of the CXCL1, CCL2, and CCL5 production by microorganisms or other stimuli/stressors will have an enormous impact on periodontal inflammation and tissue homeostasis.

During mastication but especially in orthodontic tooth movement, teeth and, therefore, the surrounding periodontal tissues are subjected to biomechanical loading. Biomechanical stimuli are critical for the maintenance, formation, and remodeling of soft and hard tissues, but it has also been demonstrated that overloading can lead to inflammation, proteolysis, and bone resorption [14, 15]. Therefore, like in periodontitis, chemokine levels are altered in periodontal tissues under biomechanical loading conditions. The potential of biomechanical forces to regulate inflammation and tissue formation is clinically used in orthodontic therapy, which aims to correct malposition of teeth.

So far, little is known whether and with what consequence microbial and biomechanical signals interact on periodontal cells and tissues. Therefore, this study was established to investigate whether the chemokines CXCL1, CCL2, and CCL5 are produced in periodontal cells and tissues and, if so, whether their levels are regulated by microbial and/or biomechanical signals in vitro and in vivo.

Materials and methods

Human gingival biopsies from periodontally healthy and periodontitis sites

The healthy gingival biopsies and the inflamed gingival tissues were obtained from different patients. Healthy gingiva (n = 6) became available during wisdom tooth surgery and tooth extraction procedures for orthodontic indications in the Department of Oral Surgery, University of Bonn. Inflamed gingival tissues were obtained from periodontitis patients (n = 6) during tooth extraction procedures. These teeth had to be removed for periodontal reasons. Approval of the Ethics Committee of the University of Bonn was obtained (#043/11), and written informed consent was provided by the patients. Exclusion criteria were presence of systemic diseases and/or smoking habits. Gingival sites with a gingival index = 0 (no clinical inflammation), periodontal probing depth ≤3 mm, no clinical attachment loss, and no radiographic bone loss were considered periodontally healthy, whereas gingival sites with a gingival index > 1, periodontal pocket depth ≥5 mm, clinical attachment loss ≥3 mm, and radiographic bone loss were considered periodontally diseased (periodontitis).

Gingival biopsies from rats subjected to experimental periodontitis and/or orthodontic tooth movement

The animal experiments were performed according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and approved by the Ethical Committee on Animal Experimentation at the School of Dentistry at Araraquara, São Paulo State University - UNESP (Protocol Number: 23/2012). Adult Holtzman rats (n = 32) of 300 g were housed in the animal facility of the university and provided standard laboratory food and water ad libitum. The rats were randomly divided into four groups: a) untreated control, b) periodontitis, c) tooth movement, d) combination of periodontitis and tooth movement. Experimental periodontitis was induced by ligatures under anesthesia with intramuscular injections of ketamine chlorhydrate 10% (0.08 mL/100 g body weight) and xylazine chlorhydrate 2% (0.04 mL/100 g body weight), as previously reported [16–18]. A cotton ligature was tied around the cervical area of the first molars in the maxilla, and the knot was placed mesially. In order to perform orthodontic tooth movement, a closed-coil nickel-titanium spring (Sentalloy®, GAC, Dentsply) providing a relatively constant force of 25 g was connected between the maxillary first molar and maxillary central incisor teeth, as previously published [16]. To prevent displacement of the 0.20-mm steel wire (CrNi, 55.01.208, Morelli, Brazil), grooves were prepared on the maxillary central incisor teeth. Afterwards, the wire in the groove was covered with a thin layer of composite resin. For placement of the spring on the maxillary first molars, composite resin was placed on the occlusal surface and, subsequently, the spring was positioned over it. The first molars in the lower jaw were removed to avoid occlusal interferences. Following 5 days of periodontitis induction, orthodontic tooth movement was initiated for 1 day and 3 days. Afterwards, 4 animals per group and time were sacrificed. Subsequently, the gingiva of the
maxillary first molars was gently harvested for RNA extraction with subsequent real-time PCR.

**Human periodontal fibroblasts**

Human periodontal ligament fibroblasts were obtained from periodontally healthy teeth, which became available during wisdom tooth surgery or tooth extraction procedures for orthodontic indications (see below). Approval of the Ethics Committee of the University of Bonn was obtained (#043/11), and written informed consent was provided by the patients. The periodontal fibroblasts were harvested from the middle third of the root surface and cultured in Dulbecco’s minimal essential medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100-U/mL penicillin and 100-µg/mL streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO₂. The periodontal fibroblasts were used between 3rd and 5th passages. Prior to the experiments, the FBS concentration was reduced to 1% and the medium was changed every second day during the experiments. To simulate periodontal infection in vitro, cells were exposed to *F. nucleatum* ATCC 25586 (OD₆₅₀ 0.05). The bacteria were suspended in phosphate-buffered saline (OD₆₅₀ = 1.0, equivalent to 1.2 × 10⁹ bacterial cells/mL) and subjected twice to ultrasonication (160 W for 15 min). Colony-forming units were determined before and after ultrasonication of the suspension. No viable bacteria were found. To mimic orthodontic tooth movement in vitro, periodontal fibroblasts were subjected to constant tensile forces (CTS) by using an established cell strain device for up to 2 days, as previously demonstrated [19, 20]. Additionally, cells were subjected simultaneously to *F. nucleatum* and CTS. Untreated cells served as controls.

**Scanning electron microscopy photographs**

Scanning electron microscopy was performed at the Center of Electron Microscopy, University Hospital of Jena, Jena (Germany), to see the attachment of *F. nucleatum* on periodontal fibroblasts. Cells were cultured on glass slides, and when a monolayer was formed, cells were exposed to *F. nucleatum* ATCC 25586 for 1 h. Afterwards, periodontal fibroblasts were fixed in 2% glutaraldehyde in cacodylate buffer for 30 min, washed twice with cacodylate buffer, and dehydrated using a 10% graded ethanol series. Critical point drying was performed, and then samples were sputter-coated with gold. Samples were evaluated using a ZEISS LEO-1530 Gemini (Carl Zeiss NTS GmbH, Oberkochen, Germany) equipped with a field emission electron gun at 10 keV.

**Gene expression of CXCL1, CCL2, and CCL5**

Total RNA extraction was carried out by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s protocol. RNA concentration was analyzed by the NanoDrop ND-2000 (Thermo Fisher Scientific, Wilmington, DE, USA) spectrophotometer, and 500 ng of total RNA was reversely transcribed using the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany) at 42 °C for 90 min followed by 85 °C for 5 min as per manufacturer’s instructions. The gene expressions of CXCL1, CCL2, and CCL5 were subsequently determined in triplicate by using QuantiTect Primers (Qiagen), SYBR Green QPCR Master Mix (Bio-Rad), and the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad). Amplification was performed under the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s, and combined annealing/extension at 60 °C for 30 s. Glyceraldehyde-3- phosphate dehydrogenase was used as a housekeeping gene, and all data were analyzed by using the comparative threshold cycle method.

**Protein levels of CXCL1, CCL2, and CCL5**

Human gingival biopsies were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 2 days, dehydrated in an ascending ethanol series (AppliChem, Darmstadt, Germany), embedded in paraffin (McCormick Scientific, Richmond, IL, USA), sectioned at 2.5 µm thickness, mounted onto glass slides (Carl Roth, Karlsruhe, Germany), and dried at 37 °C overnight. Following deparaffinization and rehydration, the sections were rinsed in PBS for 2 min. Afterwards, the endogenous peroxidase was blocked by using 0.3% methanol (AppliChem)/H₂O₂ (Merck Eurolab) solution for 5 min. The sections were then blocked with goat serum (Dako) for 20 min and incubated with rabbit anti-CXCL1, anti-CCL2, or anti-CCL5 polyclonal antibody (all Abcam; 1:100) in a humid chamber at 4 °C overnight. After washing, the sections were incubated with goat anti-rabbit IgG-HRP secondary antibody (Dako) at room temperature for 30 min. The peroxidase activity was visualized with 3,3’-diaminobenzidine chromogen (Thermo Fisher Scientific). Finally, slides were rinsed and counterstained with Mayer’s hematoxylin (Merck Eurolab) for 1 min. The images were taken with an Axioskop 2 microscope and, subsequently, analyzed with the AxioVision 4.7 software.

Protein levels of CXCL1, CCL2, and CCL5 in supernatants of periodontal fibroblasts at 1 day and 2 days were measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (LSBio, Seattle, WA, USA) according to the manufacturer’s protocol. Final absorbance was analyzed with a microplate reader (PowerWave x, BioTek Instruments, Winooski, VT, USA) at 450 nm. For data normalization, the
periodontal fibroblasts were detached after collection of the supernatants and counted with an automatic cell counter (Moelab, Hilden, Germany).

**Statistical analysis**

For statistical analysis, the IBM SPSS Statistics software (Version 22, IBM SPSS, Chicago, IL, USA) was applied. Mean values and standard errors of the mean (SEM) were calculated for quantitative data. In order to test for significant differences between the groups, the t test, Mann-Whitney U test, and ANOVA followed by the post-hoc Tukey multiple comparison tests were applied. Differences between groups were considered significant at \( p < 0.05 \).

**Results**

**Presence of CXCL1, CCL2, and CCL5 in human gingival biopsies**

First, we sought to determine if CXCL1, CCL2, and CCL5 are expressed in human gingival biopsies. As evidenced by real-time PCR, significantly (\( p < 0.05 \)) higher expression levels of CXCL1, CCL2, and CCL5 were found in the gingiva from sites of periodontitis as compared with biopsies from periodontally healthy sites (Fig. 1a–c). These findings at transcriptional level were confirmed by immunohistochemistry analysis, which demonstrated a pronounced immunostaining for CXCL1, CCL2, and CCL5 proteins in the gingiva of periodontitis specimens (Fig. 1d–f). Interestingly, the chemokines were mainly present in the subepithelial region. By contrast, clinically healthy gingiva did not show a marked presence of these chemokines (Fig. 1d–f).

**Presence of CXCL1, CCL2, and CCL5 in gingival biopsies of rats with experimental periodontitis and/or orthodontic tooth movement**

Since the aforementioned analyses revealed that the levels of all chemokines were increased at sites of periodontitis, we wondered if biomechanical forces could regulate the bacteria-induced chemokine expressions. To study the interactions of microbial and biomechanical signals in a controlled environment, an animal model was used. The rats were subjected to experimental periodontitis and/or orthodontic tooth movement, untreated rats served as control. As shown in Fig. 2a–c, periodontitis resulted in significantly (\( p < 0.05 \)) enhanced expression levels of CXCL1, CCL2, and CCL5 in the gingival biopsies. However, when the sites of experimental periodontitis were also subjected to orthodontic tooth movement, the gingival levels of all chemokines were significantly (\( p < 0.05 \)) lower as compared with the periodontitis group, demonstrating that the orthodontic forces counteracted the bacteria-induced upregulation of CXCL1, CCL2, and CCL5 (Fig. 2a–c).

**Regulation of CXCL1, CCL2, and CCL5 by \( F. \) nucleatum and/or biomechanical forces in fibroblasts**

Since \( F. \) nucleatum is a pathogen which plays a critical role in both gingivitis and periodontitis, periodontal fibroblasts were exposed to this bacterium in further in vitro experiments (Fig. 3). As depicted in Fig. 4a, \( F. \) nucleatum caused a significant (\( p < 0.05 \)) upregulation of CXCL1 in the cells at 1 day, as analyzed by real-time PCR. However, when these cells were subjected simultaneously to \( F. \) nucleatum and CTS, the \( F. \) nucleatum-induced upregulation of CXCL1 was significantly (\( p < 0.05 \)) inhibited (Fig. 4a). Like CXCL1, CCL2 and CCL5 were significantly (\( p < 0.05 \)) increased in the presence of \( F. \) nucleatum (Fig. 4 b and c). Furthermore, the upregulated CCL2 and CCL5 expressions were significantly (\( p < 0.05 \)) diminished again, when the fibroblasts were treated with a combination of \( F. \) nucleatum and CTS (Fig. 4 b and c). As evidenced by ELISA, exposure of the cells with \( F. \) nucleatum also resulted in significantly (\( p < 0.05 \)) enhanced protein levels of CXCL1, CCL2, and CCL5. Moreover, application of CTS to the cells counteracted the stimulatory action of \( F. \) nucleatum on CXCL1, CCL2, and CCL5 proteins, confirming and expanding the findings at transcriptional level (Fig. 4d–f).

**Discussion**

This study provides original evidence in vitro and in vivo that the chemokines CXCL1, CCL2, and CCL5 are regulated by both microbial and biomechanical signals in periodontal cells and tissues. Furthermore, our study revealed that biomechanical forces can counteract the stimulatory actions of \( F. \) nucleatum on these chemokines. Therefore, biomechanical loading might aggravate periodontal infection by compromising the recruitment of immunoinflammatory cells.

Periodontitis is characterized by a chronic inflammation caused by subgingival microorganisms [2]. The aim of the inflammatory host response is to eliminate or at least to reduce the pathogenic bacteria, thereby protecting the periodontal cells, tissues, and structure. In order to recruit immunoinflammatory cells to the site of the microbial attack and tissue destruction, chemokines, such as CXCL1, CCL2, and CCL5, are required. Whereas CXCL1 is a strong chemoattractant for neutrophils, CCL2 and CCL5 mainly promote the recruitment of monocytes, macrophages, and lymphocytes [13]. Several studies have clearly demonstrated that these cell types and their subsets have defined roles in the battle between the host and microorganisms in periodontitis [21, 22].
Our study proves that CXCL1, CCL2, and CCL5 are of utmost importance in the pathogenesis of periodontal diseases.

They were upregulated in human gingiva from sites of periodontitis, in inflamed gingiva from rats and also in periodontal...
fibroblasts exposed to the periodontopathogen *F. nucleatum*. These transcriptional results were further supported by findings at protein level, as evidenced by immunohistochemistry and ELISA. Interestingly, the role of the aforementioned chemokines has also been addressed by other investigators. For example, increased CXCL1, CCL2, and CCL5 levels have been found in gingival crevicular fluid (GCF) from periodontally diseased sites as compared with healthy sites [10, 23, 24]. Moreover, elevated CCL2 levels have been observed in human gingival tissues affected by periodontitis [25, 26]. These observations concur with our findings, indicating that periodontal cells and tissues produce enhanced levels of CXCL1, CCL2, and CCL5 in the presence of periodontal infection and may therefore contribute to elevated levels of these chemokines in GCF. Notably, increased serum levels of CXCL1 and CCL5 have been demonstrated in experimental periodontitis in mice [6, 27]. Whether the locally produced levels of CXCL1, CCL2, and CCL5 from the periodontal tissues contribute to the elevated serum levels of these chemokines should be further analyzed.

Few studies have also focused on the actions of mechanical forces on the chemokine production in periodontal cells and tissues. Periodontal cells subjected to compressive forces for 24 h produced higher CCL2 levels as controls [28]. Increased CCL2 periodontal tissue levels following mechanical force application were also observed in animal models [28, 29]. These findings are in contrast to our results, which could be due to different reasons. For example, their in vivo studies focused on CCL2 in periodontal ligament of the compression site, whereas we examined CCL2 in gingiva without restriction to a particular site. In vitro, we subjected periodontal ligament cells to tensile strain in contrast to compressive forces. In humans, the effect of mechanical forces on CCL2 and CCL5 has been studied during orthodontic tooth movement and yielded controversial results [30–32]. The regulation of these chemokines may depend on several parameters, such as duration, magnitude, and type of force application as well as the biological material evaluated.

In our study, the bacteria-induced upregulation of CXCL1, CCL2, and CCL5 was surprisingly counteracted by biomechanical forces in vitro and in vivo, suggesting that biomechanical loading may compromise a sufficient recruitment of immunoinflammatory cells in periodontitis. This mechanism may explain how occlusal overloading may aggravate periodontitis, as it has clearly been shown in animals [33]. Interestingly, CXCL1 and CCL5 promote migration of gingival fibroblasts and CCL5 stimulates proliferation of these cells [34], indicating that these chemokines affect not only neutrophils, monocytes/macrophages, and lymphocytes but also periodontal cells.

Although it was easy to obtain human gingival biopsies from sites of periodontitis and periodontal health, gingival tissues from sites affected by both periodontitis and orthodontic forces become hardly available. Therefore, an animal model was chosen to study the interaction of periodontitis and biomechanical/orthodontic forces. In this model, periodontitis was induced by cotton ligatures around the maxillary first molars, which promoted the accumulation of a complex bacterial biofilm and bone resorption, as previously shown [16]. Extrapolating data from rats to humans has to be done with caution. However, a study by Hyde et al. on the characterization of the rat oral microbiome has demonstrated that there are sufficient similarities in oral microbiome structure and physiological effects to justify the use of rat models, even if there are limitations [35]. We also wanted to study the interactions between microbial and mechanical signals at cellular levels and therefore exposed periodontal fibroblast to both *F. nucleatum* and CTS, which was applied by the use of an established cell strain device. However, during orthodontic tooth movement and mastication, periodontal tissues are subjected to complex mechanical loading. Whether compressive, hydrostatic, and shear forces as well as their combinations exert similar regulatory effects on CXCL1, CCL2, and...
CCL5, as observed for tensile strain in the present investigation, needs to be elucidated in further studies. The magnitude of the applied tensile force was physiologically relevant and also chosen in our previous studies.

In our in vitro experiments, the periodontal fibroblasts were treated with \textit{F. nucleatum}, because this Gram-negative, anaerobic periodontopathogen serves as a bridge between early and late colonizers during biofilm formation, can invade host cells, and has a proven etiological role in gingivitis and periodontitis \cite{3, 4, 36}. Nevertheless, periodontitis not only is caused by a single bacterium but also is a highly complex biofilm \cite{37}. Future studies should therefore focus on the regulatory actions of other pathogenic microorganisms and their combinations on CXCL1, CCL2, and CCL5.

Since \textit{F. nucleatum} is strongly involved in periodontal diseases, this microorganism was used to simulate periodontal infection in our in vitro study. Nevertheless, \textit{Porphyromonas gingivalis} is another important and well-studied periodontopathogen. Intriguingly, \textit{P. gingivalis} and \textit{Escherichia coli} have also been shown to enhance CXCL1,
CCL2, and CCL5 expressions in human periodontal cells [38–42]. By demonstrating that these chemokines are upregulated in response to microbial stimuli, these studies support our in vitro findings. In our experiments, a suspension of F. nucleatum, which was exposed to intensive ultrasonication before application, was used. Therefore, it can be assumed that the suspension mainly contained disrupted cell wall particles with a high amount of lipopolysaccharides.

Future studies should also unravel the interactions between the microbial and mechanical signals at receptor level and within the cells. In addition, it would be intriguing to know if microbial and mechanical signals also interact on the regulation of chemokine receptors in periodontal cells and tissues. Moreover, periodontal cells with a fibroblast-like phenotype were used in our in vitro experiments. However, if other periodontal cell phenotypes, such as osteoblasts and cementoblasts, exert similar effects on CXCL1, CCL2, and CCL5 remains to be studied.

Conclusions

In summary, our in vitro and in vivo investigations have shown that the chemokines CXCL1, CCL2, and CCL5 are regulated by both bacterial and mechanical signals in periodontal cells and tissues. Additionally, our study revealed that biomechanical forces can inhibit the stimulatory actions of F. nucleatum on CXCL1, CCL2, and CCL5. Therefore, biomechanical loading may aggravate periodontal infection by compromising the critical recruitment of immunoinflammatory cells.

Acknowledgments The authors would like to thank Mrs. Ramona Menden, Mrs. Silke van Dyck, Mrs. Jana Marciniak, Prof. Sören Jepsen, and Prof. Gerhard Wahl for their valuable support.

Authors’ contributions Defining the study aims: Birgit Rath-Deschner and Andressa V. B. Nogueira; coordination of collaboration: Andreas Jäger and James Deschner; planning the experiments: Birgit Rath-Deschner, Andressa V. B. Nogueira, Joni A. Cirelli, Anna Damanaki, James Deschner, Marjan Nokhbehseim, and Svenja Memmert; growing the bacteria and preparing the bacterial lysate: Sigrun Eick; performing the experiments: Birgit Rath-Deschner, Andressa V. B. Nogueira, Anna Damanaki, Marjan Nokhbehseim, and Svenja Memmert; monitoring/supervising the experiments: Andreas Jäger, James Deschner, and Joni A. Cirelli; analyzing and discussing the data: Birgit Rath-Deschner, Andressa V. B. Nogueira, Anna Damanaki, Svenja Memmert, James Deschner, Werner Götz, and Andreas Jäger; creating the figures: Birgit Rath-Deschner and Andressa V. B. Nogueira. All authors contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Funding information Open Access funding provided by Projekt DEAL. This study was supported by the German Society of Orthodontics (DGKFO), the São Paulo Research Foundation, Brazil (FAPESP: 2014/20715-7, 2017/07137-2) and the German Research Foundation (DE1593/5-1), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brazil within the Program Capes/Probral (ID 88881.144012/2017-01) and German Academic Exchange Service, DAAD, Germany (ID 57391253).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of the University of Bonn (043/11) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

All procedures performed in this study were in accordance with the ethical standards of the Ethical Committee for Animal Use (CEUA) of the School of Dentistry at Araraquara – UNESP (23/2012) and performed in accordance with the guideline from the Brazilian College for Animal Experimentation (COBEA). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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