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The essential role of toll like receptor-4 in the control of *Aggregatibacter actinomycetemcomitans* infection in mice

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Abstract

Objective: *Aggregatibacter actinomycetemcomitans* is an oral Gram-negative bacterium that contributes to periodontitis progression. Isolated antigens from *A. actinomycetemcomitans* could be activating innate immune cells through Toll-like receptors (TLRs). In this study, we evaluated the role of TLR4 in the control of *A. actinomycetemcomitans* infection.

Material and Methods: We examined the mechanisms that modulate the outcome of *A. actinomycetemcomitans*-induced periodontal disease in TLR4$^{-/-}$ mice. The production of cytokines was evaluated by ELISA. The bacterial load was determined by counting the number of colony-forming units per gram of tissue.

Results: The results showed that TLR4-deficient mice developed less severe periodontitis after *A. actinomycetemcomitans* infection, characterized by significantly lower bone loss and inflammatory cell migration to periodontal tissues. However, the absence of TLR4 facilitated the *A. actinomycetemcomitans* dissemination. Myeloperoxidase activity was diminished in the periodontal tissue of TLR4$^{-/-}$ mice. We observed a significant reduction in the production of tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-1β in the periodontal tissue of TLR4$^{-/-}$ mice.

Conclusion: The results of this study highlighted the role of TLR4 in controlling *A. actinomycetemcomitans* infection.

Key words: gingival IL-1β; gingival myeloperoxidase; gingival TNF-α; periodontal disease

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Periodontal diseases are chronic inflammatory diseases of the attachment structures of the teeth. The periodontal biofilm hosts a wide diversity of potentially harmful bacterial species, which trigger immune responses that can lead to tissue destruction (Lappin et al. 2003, Berglundh & Donati 2005, Taubman et al. 2005). *Aggregatibacter actinomycetemcomitans* is an oral Gram-negative bacterium and is related to aggressive periodontitis (Aberg et al. 2009, Faveri et al. 2009) and other systemic diseases, such as endocarditis as well as lung, skin and urinary tract infections (van Winkelhoff & Slots 1999, Henderson et al. 2003). The lipopolysaccharide (LPS) of *A. actinomycetemcomitans* is a strong virulence factor; it exhibits various activities that are closely correlated with periodontal disease, such as induction of inflammatory cytokines in human gingival fibroblast cultures (Gutierrez-Venegas et al. 2006, Patil et al. 2006, Sun et al. 2008) and bone resorption activity (Garlet et al. 2007, 2008). LPS may elicit the activation of

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many inflammatory cells as epithelial cells, fibroblasts, neutrophils, macrophages and other cells through activation of Toll-like receptor-4 (TLR4) (Uchida et al. 2001, Sosroseno et al. 2002, Sosroseno & Herminajeng 2002, Remer et al. 2003, Shin et al. 2008). TLRs belong to a family of leucine-rich repeat (LRR) proteins that are either expressed at the cell surface or at the intracellular compartments of inflammatory cells. Phagocytes activation through TLR signalling leads to increased phagocytosis and cytokine production (Aderem & Ulevitch 2000, Elson et al. 2007).

The signalization via TLR4 induces the activation of several innate pathways, such as phagocytosis, overexpression of pro-inflammatory chemokines, cytokines and co-stimulatory molecules, which lead to initiation of the inflammatory response (Underhill et al. 1999, Madianos et al. 2005, Elson et al. 2007). Specifically, TLR4 is involved in monocytes and neutrophils recruitment and activation (Netea et al. 2002, Burch et al. 2006). TLR4 is expressed in almost all cells of periodontal tissues and is associated with the development of periodontal disease (Kajita et al. 2007, Mahanonda & Pichyangkul 2007, Nakamura et al. 2008, Sun et al. 2008).

Despite the clear association between TLR4 and the recognition of *A. actinomycetemcomitans* (Kikkert et al. 2007) the molecular mechanisms connecting this receptor to the inflammatory reaction in periodontal tissues induced by this bacterium have not been established. The aim of this study was to investigate the mechanisms that modulate the outcome of *A. actinomycetemcomitans*-induced periodontal disease in mice.

Materials and Methods

Animals

Experimental groups consisting of 6-week-old male wild-type C3H/HePas mice (WT) and TLR4-deficient mice C3H/HeJ ($TLR4^{-/-}$) were bred (breeding pairs obtained from Jackson Laboratory, Bar Harbor, ME, USA) and maintained in the animal facilities of the Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Brazil. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use (#002/2008).

Bacterial growth

A. actinomycetemcomitans (strain JP2) was cultured in tryptic soy agar broth supplemented with 0.6% yeast extract (TSBYE, BD Biosciences, San Jose, CA, USA) at 37°C in a humidified, 10% CO_2 atmosphere. Bacterial morphology was confirmed by Gram method and by visualization in a stereomicroscope (MS 23358; Wild Heerbrugg, Gais, Switzerland). Colonies purity was confirmed by catalase reaction and polymerase chain reaction (PCR). Bacterial concentrations used in experiments were determined with MacFarland Standards.

Experimental periodontal disease

Periodontal infection was achieved by oral delivery of 1×10^9 colony-forming units (CFU) of *A. actinomycetemcomitans* diluted in 50 μl of sterile phosphate-buffered saline (PBS) containing 2% carboxymethylcellulose, into the oral cavity of mice with a micropipette, as previously described (Garlet et al. 2007). After 24 and 48 h, this procedure was repeated. Negative controls included non-infected and sham-infected mice; the later received 50 μl of PBS containing 2% carboxymethylcellulose solution. After infection, the mice were periodically weighed and evaluated regarding survival. Sixty days after the infection, animals were euthanized and the organs (periodontal tissue, liver, kidney, spleen, heart and lungs) were collected for molecular and histopathological analyses. The presence of *A. actinomycetemcomitans* in periodontal tissues was confirmed by PCR and by the number of CFUs observed over TSBYE plates.

Quantification of alveolar bone loss

Evaluation of the extent of alveolar bone loss was performed as described previously (Garlet et al. 2007). Alveolar bone loss was measured at 3, 7, 10, 30 and 60 days after the last *A. actinomycetemcomitans* oral inoculation. The maxillae were hemisected, exposed overnight in 3% hydrogen peroxide and defleshed mechanically. The palatal surfaces of the molars were photographed at × 25 magnification using a stereomicroscope, with the occlusal face of the molars positioned parallely to the base. The images were digitized and analysed using Image J 1.36b (Wayne Rasband, National Institutes of Health, USA). Quantitative analysis was used for the measurement of the area between the cementum–enamel junction and the alveolar bone crest in the three posterior teeth, in arbitrary units of area. Ten animals per group were analysed and, for each animal, the alveolar bone loss was defined as the average cementum–enamel junction/alveolar bone crest area in all molars from both right and the left arches.

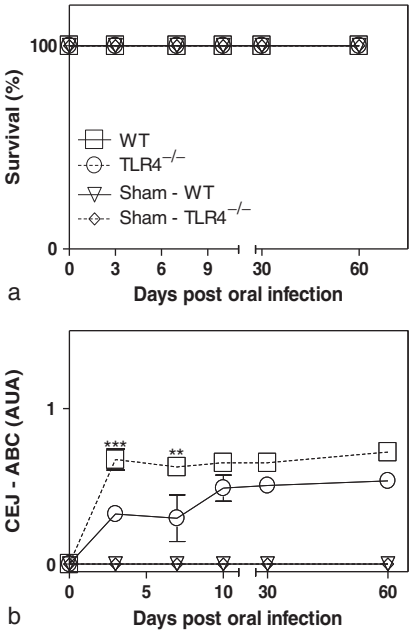

Fig. 1. Alveolar bone loss induced by *Aggregatibacter actinomycetemcomitans* in WT and $TLR4^{-/-}$ mice. WT (□) and $TLR4^{-/-}$ (○) mice were submitted to oral inoculation with *A. actinomycetemcomitans*. (a) Mouse survival during the course of experimental periodontal disease. (b) The figure represents the measurements of cementum–enamel junction (CEJ) to the alveolar bone crest (ABC) area in the palatine face of maxillary molars. The results are represented as mean ± SD of the CEJ–ABC (10 animals/group) and are representative of three independent experiments. The data were analysed by two-way ANOVA followed by the Bonferroni test. $^{***}p<0.001$ and $^{**}p<0.01$ compared with control group.

Histological analysis

Histological analyses were carried out as described previously (Garlet et al. 2007). Briefly, the periodontal tissues obtained from four animals, randomly selected from each group, were sacrificed at 0 and 60 days after infection.

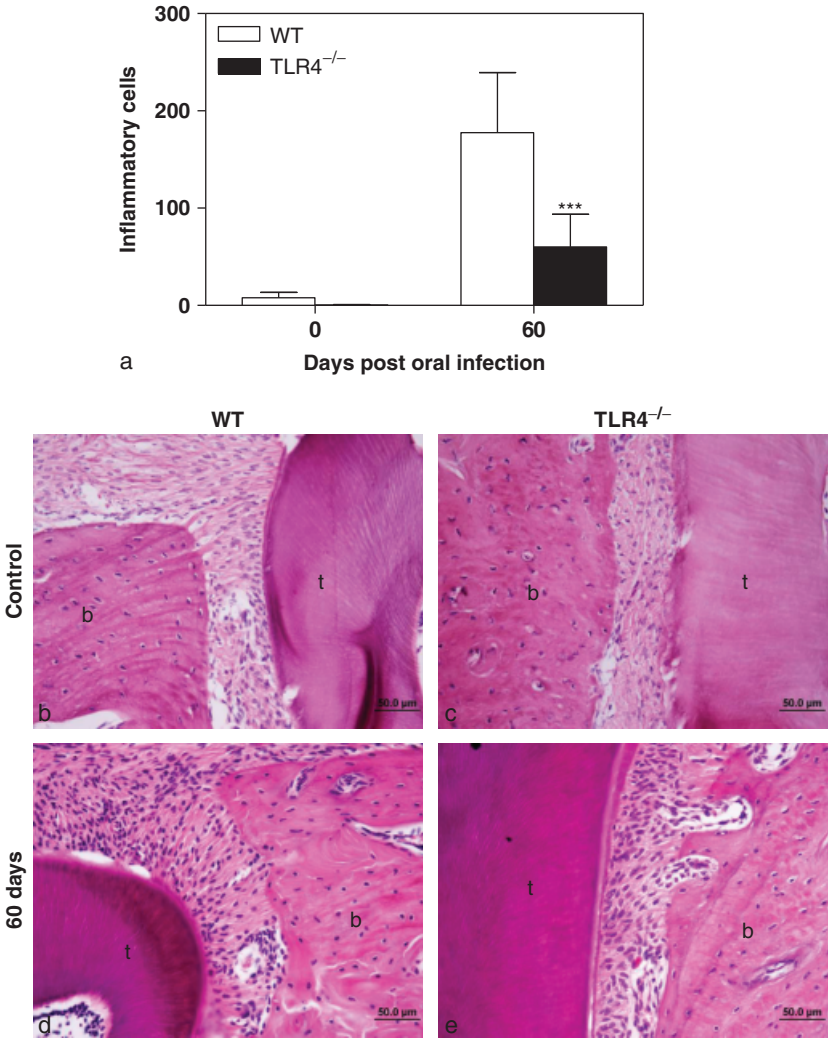

Fig. 2. Inflammatory reaction induced by *Aggregatibacter actinomycetemcomitans* in WT and TLR4$^{-/-}$ mice. WT (□) and TLR4$^{-/-}$ (■) mice were submitted to oral inoculation with *A. actinomycetemcomitans*. (a) Inflammatory cells were quantified in histological samples. (b–e) Histological analysis was evaluated 60 days after infection (hematoxylin and eosin staining, × 400 magnification). Bone (b) and tooth (t) areas are indicated. The data were analysed by *t* test. $^{***}p<0.001$ compared with control group.

The periodontal tissues obtained were then fixed with 4% paraformaldehyde in PBS, pH 7.4, for 12 h at room temperature. The specimens were thoroughly demineralized in 10% ethylenediaminetetraacetic acid disodium salt for 1–2 weeks. The decalcified mouse periodontal tissues were trimmed, dehydrated in graded ethanol and embedded in paraffin. Serial sections (5 µm) were cut and mounted on glass slides precoated with 0.1% poly-L-lysine (Sigma, St. Louis, MS, USA). Histological assessment was carried out following routine haematoxylin and eosin staining, as well as inflammatory cells were quantified at histological slices (10 areas of periodontal ligament and cervical gingival area were randomly chosen) using Axiovision 4.5 (Carl Zeiss, Jena, Germany).

Dissemination of *A. actinomycetemcomitans*

In order to determine the growth and dissemination of *A. actinomycetemcomitans*, the amount of CFUs recovered from the gingival tissue, lymph nodes, spleen, heart, lung, liver and kidney were analysed 60 days after infection. The organs were removed, weighed and homogenized in PBS using tissue grinders, and the samples (100 µl) were diluted in 900 µl of PBS. Aliquots of 100 µl of each sample were dispensed into Petri dishes containing TSBYE. The plates were incubated at 37°C, the colonies counted 7 days later and the number of CFUs per gram of tissue (organs) was calculated.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity in homogenized periodontal tissue was measured by enzymatic reaction through the absorbance at 450 nm, as previously described (Garlet et al. 2007).

Cytokine production

Measurements of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β in periodontal tissues were performed as described previously (Garlet et al. 2007). The palatal gingival tissue from WT or TLR4$^{-/-}$ *A. actinomycetemcomitans*-infected mice was collected through transversal incision at the distal surface of third molar teeth and palatal surface from incisors. The production of TNF-α and IL-1β was determined by ELISA using commercially available kits (OptEIA™, BD Biosciences) as follows: TNF-α (sensitivity >3.4 pg/ml) and IL-1β (>4.2 pg/ml). All assays were carried out according to the manufacturer's instructions and performed in triplicate. Tissue protein content was quantified with Quick Start™ Bradford Protein assay kit (Bio-Rad, Hercules, CA, USA) and the concentrations of each cytokine were expressed as picogram per milligram of protein (for three independent experiments).

Statistical analysis

The results are expressed as the mean standard deviation (SD). Statistical analysis was performed using analysis of variance (two-way ANOVA) followed by Bonferroni post-test (PRISM Software; GraphPad, San Diego, CA, USA). Student's *t* test was used to compare two groups. Values of $p<0.05$ were considered to be statistically significant.

Results

A. actinomycetemcomitans induces less severe periodontal disease in TLR4$^{-/-}$ mice

First, we evaluated the role of TLR4 in the modulation of experimental periodontal disease severity in mice infected with *A. actinomycetemcomi-*

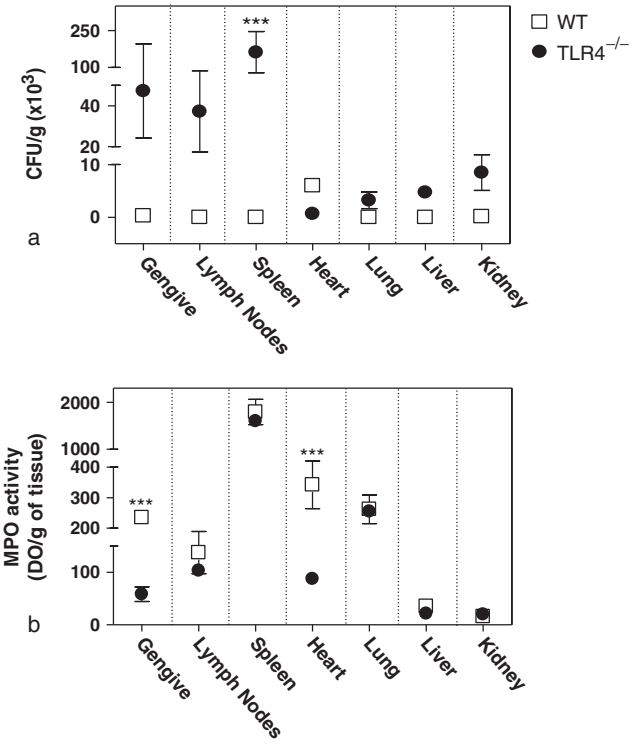

Fig. 3. Absence of TLR4 influences the myeloperoxidase (MPO) activity and *Aggregatibacter actinomycetemcomitans* dissemination. WT (□) and TLR4$^{-/-}$ (●) mice were submitted to oral inoculation with *A. actinomycetemcomitans*. Gingival tissue, lymph node, spleen, liver, heart, lung and kidney were collected to assess the bacteria load (a) and (b) MPO activity after 60 days post-oral inoculation with *A. actinomycetemcomitans*. The results are representative of three independent experiments. The data were analysed by two-way ANOVA followed by the Bonferroni test. ***$p<0.001$ compared with control group.

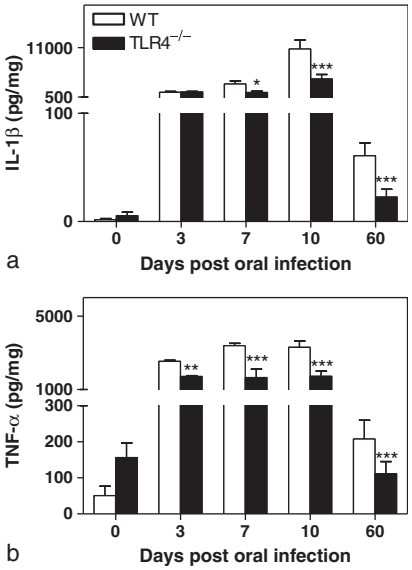

Fig. 4. Absence of TLR4 alters cytokines production involved with *Aggregatibacter actinomycetemcomitans* immune response. WT (□) and TLR4$^{-/-}$ (■) mice were submitted to oral inoculation with *A. actinomycetemcomitans* and during the course of experimental periodontal disease periodontal tissues were collected, homogenized, and the levels of IL-1β (a) and TNF-α (b) were evaluated by ELISA. The results were normalized for the protein content of each sample and are expressed as means ± SD for three experiments. Data were analysed by *t* test. ***$p<0.001$, **$p<0.01$, *$p<0.05$ compared with control group.

tans. The infection did not result in alteration of weight gain and mortality of TLR4$^{-/-}$ mice (Fig. 1a). TLR4$^{-/-}$-infected mice presented significantly lower bone loss when compared with WT mice (Fig. 1b). Histological analysis of periodontal tissues (gingival area and periodontal ligament) showed a considerable inflammatory reaction in WT mice when compared with TLR4$^{-/-}$ mice (Fig. 2). The TLR4$^{-/-}$ inflammatory infiltrate was mainly characterized by macrophages and neutrophils and lymphocytes were also observed. This finding became more evident at 60 days post-infection, and was significantly lower than WT mice (Fig. 2a).

Absence of TLR4 influences the MPO activity and *A. actinomycetemcomitans* dissemination

TLR4$^{-/-}$ mice had a significantly higher bacterial load in periodontal tissues (Fig. 3a), and lower levels of MPO activity when compared with WT-infected mice (Fig. 3b). In addition to the periodontal tissue, *A. actinomycetemcomitans* was found in the lymph nodes, spleen, lungs, liver and kidney of TLR4 mice, while in the WT mice this pathogen was restricted to the heart (Fig. 3a). Uninfected and sham-infected mice presented no periodontal or systemic alterations in all analyses (Fig. 3).

Absence of TLR4 influences the production of cytokines in *A. actinomycetemcomitans*-infected mice

Next, we evaluated the levels of IL-1β and TNF-α produced in the gingival tissue of TLR4$^{-/-}$ and WT mice (Fig. 4). The results show significantly higher production of IL-1β (Fig. 4a) and TNF-α (Fig. 4b) in the gingival tissue of WT-infected mice as compared with the samples from TLR4$^{-/-}$-infected mice at 7, 10 and 60 days from infection. At the third day after oral infection, no difference in IL-1β production was observed in periodontal tissue from WT and TLR4$^{-/-}$ mice.

Discussion

The recognition of LPS by innate immune cells occurs mainly through TLR4, and this activation leads to cytokine and chemokine production that is related with neutrophils and macrophages chemotaxis (Jerala 2007). In this study, we demonstrated that TLR4-deficient mice (TLR4$^{-/-}$) infected with *A. actinomycetemcomitans* exhibit decreased inflammatory cell migration and alveolar bone resorption when compared with WT mice. Accordingly, a previous study has demonstrated that mice lacking TLR4 receptor presented reduced inflammatory reaction and bone loss in response to LPS from *A. actinomycetemcomitans* applied on the gingival tissue (Nakamura et al. 2008).

The absence of TLR4 resulted in the impairment of the protective immunity

against *A. actinomycetemcomitans*, as demonstrated by the higher bacterial load in the periodontium and bacterial dissemination. Indeed, *A. actinomycetemcomitans* JP2 strain is characterized by its high virulence, invasiveness and leucotoxic activity (Henderson et al. 2003, Dileepan et al. 2007, Haubek et al. 2007), even though more pronounced disease had been observed using adherent *A. actinomycetemcomitans* strains in rat model (Fine et al. 2001, Schreiner et al. 2003). Interestingly, before being recognized as a periodontopathogen, *A. actinomycetemcomitans* was described in systemic and disseminated infections, particularly in endocarditis and pulmonary lesions (Brisseau et al. 1988, Kaplan et al. 1989, van Winkelhoff & Slots 1999). Our results corroborate such findings and showed that in WT mice the *A. actinomycetemcomitans* detection was restricted to the heart, while in TLR4$^{-/-}$ mice this bacterium was detected in the periodontal tissues, lungs, lymph nodes, spleen, liver and kidney, which corresponded to the places where modest inflammatory infiltrates were also detected. Although *A. actinomycetemcomitans* was not observed in periodontal tissues from WT mice, these animals presented higher MPO activity when compared with TLR4$^{-/-}$ mice, which may have protected the WT mice from the dissemination of the pathogen. MPO is an anti-microbial enzyme involved with the production of several potent anti-microbial reactive oxygen species. Its lower activity in periodontal tissues has been correlated with impaired response against *A. actinomycetemcomitans* infection (Garlet et al. 2007). MPO activity is directly related to neutrophils infiltration (Hsieh et al. 2008), and the lower MPO activity detected in TLR4$^{-/-}$-infected mice could indicate a reduced influx of neutrophils in these animals. These data suggested that TLR4 deficiency also impairs the local host response against this periodontopathogen. In spite of the bacteria dissemination, we did not observe pronounced death of TLR4$^{-/-}$ mice after 60 days of experimental periodontitis. Therefore, the absence of this receptor impaired the host response, but did not influence mice survival after *A. actinomycetemcomitans* infection.

Periodontal disease is a chronic inflammatory condition recognized as the most prevalent cause of tooth loss in humans (Dixon et al. 2004, Burns et al. 2006). It involves genetic factors that determine the host immune response against microorganisms, environmental factors and plaque-associated bacteria, like *A. actinomycetemcomitans* and *Porphyromonas gingivalis* (Kinane & Lappin, 2001, Gemmell & Seymour, 2004). Induced periodontitis is characterized by an intense increase in the inflammatory reaction, and the alveolar bone loss is associated with augmented expression of IL-1β, TNF-α and IFN-γ (Assuma et al. 1998, Garlet et al. 2008, Kelk et al. 2008, Polak et al. 2009). In fact, orally infected TLR4$^{-/-}$ mice presented a significant decrease in alveolar bone loss resorption after challenge with *A. actinomycetemcomitans* in comparison with WT mice. Hence, this lower bone loss was accompanied by a reduced inflammatory cell migration and inflammatory cytokine production. Such decreased inflammatory reaction associated with the decreased MPO activity possibly resulted in the less severe periodontitis phenotype presented by TLR4$^{-/-}$ mice. The role of TLR4 to determine protection or susceptibility in periodontal disease is an issue of recent interest. Polymorphisms in TLR4 and CD14 genes may predispose periodontal patients to severe forms of this disease (Fukusaki et al. 2007, James et al. 2007, Ozturk & Vieira 2009). In accordance, TLR4-deficient mice present reduced bone loss after Gram-negative bacteria or LPS inoculation (Hou et al. 2000, Nakamura et al. 2008). In fact, LPS cannot induce osteoclast differentiation from monocytes in the absence of TLR4 (Ito et al. 1996).

Our results demonstrate that the absence of TLR4 signalization is associated with lower inflammatory cell migration and periodontal tissues destruction. Such absence of TLR4 activation leads to impaired MPO activity in the periodontium and possibly facilitates systemic bacteria dissemination and proliferation. This knowledge is important in the understanding of bacterial recognition and development of strategies to attenuate periodontal tissue destruction without impairing the host response against periodontopathogens infection.

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Clinical Relevance

Scientific rationale for the study: LPS, virulence factor of Gram-negative bacteria, is recognized by host cells via TLR4. Thus, information regarding the participation of TLR4 in the recognition of *A. actinomycetemcomitans* may provide important information about the development and progression of periodontal disease.

Principal findings: The absence of TLR4 reduced bone loss and pro-inflammatory cytokine production. However, our results showed that TLR4 signals are important to control *A. actinomycetemcomitans* dissemination.

Practical implications: Our results demonstrate that the abrogation of TLR4 influences the host response against *A. actinomycetemcomitans*. TLR4 might be an important therapeutic target in order to avoid periodontal disease progression.