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Cytokine pattern determines the progression of experimental periodontal disease induced by *Actinobacillus actinomycetemcomitans* through the modulation of MMPs, RANKL, and their physiological inhibitors

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Objective: Inflammatory and immune reactions raised in response to periodontopathogens are thought to trigger periodontal tissue destruction. We therefore investigated the expression of matrix metalloproteinases (MMPs) and the osteoclastogenic factor RANKL (receptor activator of nuclear factor-κB ligand), their respective inhibitors TIMPs (tissue inhibitors of metalloproteinases) and OPG (osteoprotegerin) and their possible correlation with the expression of inflammatory and regulatory cytokines in the course of experimental periodontal disease in mice.
Methods: We characterized the time course of leukocyte migration and alveolar bone loss in C57BL/6 mice infected with *Actinobacillus actinomycetemcomitans*. Quantitative polymerase chain reaction (RealTime PCR) and ELISA were performed to determine the expression of MMPs, TIMPs, RANKL, OPG and cathepsin K, interleukin-1β, tumor necrosis factor-α, interferon-γ, interleukin-12, interleukin-4 and interleukin-10 in periodontal tissue samples harvested throughout the course of experimental disease.
Results: Oral inoculation of *A. actinomycetemcomitans* results in an intense and widespread migration of leukocytes to the gingival tissues, besides marked alveolar bone resorption. Our data also demonstrate two distinct patterns of MMP/TIMP and RANKL/OPG expression in the course of experimental periodontal disease. The expression of MMPs (MMP-1, 2 and 9) and RANKL was correlated with the expression of interleukin-1β, tumor necrosis factor-α and interferon-γ, in a time period characterized by the intense increase of inflammatory reaction and alveolar bone loss. On the other hand, interleukin-4 and interleukin-10 were associated with higher expression of TIMPs (TIMP 1, 2 and 3)

Key words: *Actinobacillus actinomycetemcomitans*; cytokines; experimental periodontal disease; matrix metalloproteinases; RANKL

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and OPG, with a lower expression of MMPs and RANKL, and with reduced rates of increase of cellular infiltration in periodontal tissues and alveolar bone loss.
Conclusions: It is possible that the pattern of cytokines produced in periodontal tissues determines the progression and the severity of experimental periodontal disease, controlling the breakdown of soft and bone tissues through the balance between MMPs/TIMP and RANKL/OPG expression in gingival tissues.

Periodontal diseases, chronic inflammatory diseases of the attachment structures of the teeth, are one of the most significant causes of tooth loss in adults and the most prevalent form of bone pathology in humans, besides being a modifying factor of the systemic health of patients.

The bacterial biofilm attached to the tooth surface triggers an intense inflammatory reaction, generation of proteases that degrade extracellular matrix and resorption of alveolar bone, leading to the irreversible loss of tissue attachment in the periodontium (4, 23).

Among host proteases that target the extracellular matrix, matrix metalloproteinases (MMPs, a family of zinc- and calcium-dependent proteases) play a role in both degradation and remodeling of matrix proteins during different physiological and pathological processes (7). MMPs comprise four major subclasses based on their substrate specificity and sequence homology:

- collagenases such as MMP-1 or interstitial collagenase, which are active against fibrillar collagen;
- gelatinases, also called type IV collagenases (A or MMP-2 and B or MMP-9), which present high activity against denatured collagens;
- stromelysins, which degrade noncollagen components of extracellular matrix;
- membrane-type matrix metalloproteinases (7).

The activation of MMPs is regulated by a group of endogenous proteins named tissue inhibitors of metalloproteinases (TIMPs), which are each capable of inhibiting almost every member of the MMP family in a nonspecific manner (5). Usually, the TIMPs are in balance with the MMPs and matrix is remodeled in a highly regulated fashion. However, in many disease processes the levels of MMPs are elevated without a concomitant increase in TIMPs, resulting in tissue destruction (32). It is thought that MMPs and TIMPs are involved in the physiological turnover of periodontal tissues, and MMPs appear to be involved in tissue destruction in periodontal diseases (6, 15, 33, 50). However, there are contradictory results regarding the balance of MMPs/TIMPs in pathological vs. healthy gingival samples (1, 9, 13, 18, 24, 30), and their contribution towards the pathogenesis of periodontitis is not completely understood.

In addition to the destruction of connective tissue, alveolar bone loss is a key event in periodontal diseases. The integrity of bone tissues depends on maintaining a delicate equilibrium between bone resorption by osteoclasts and bone formation by osteoblasts. RANKL (receptor activator of nuclear factor-κB ligand), its cellular receptor, RANK, and the decoy receptor, osteoprotegerin (OPG) have been identified as the key molecular regulation system for bone remodeling. RANKL is the main stimulatory factor for the differentiation and activation of osteoclasts. The effects of RANKL are counteracted by OPG, which strongly inhibits bone resorption by preventing RANK–RANKL engagement (19, 43). The RANK system also contributes to bone resorption by inducing the expression of cathepsin K, a cysteine proteinase produced by activated osteoclasts and involved in bone matrix solubilization (43). An imbalance in this system is a pivotal component of the etiology of some bone disorders (36, 37). In periodontal disease, increased levels of RANKL are found in diseased tissues, and their balance with OPG expression is supposed to determine disease severity (8, 13, 27, 29, 45, 49).

It is also important to establish the factors that regulate the breakdown of homeostasis of connective and osseous tissue that takes place in periodontal disease. Inflammatory mediators, such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α, and the Th1-type cytokine interferon (IFN)-γ, have been described as positive regulators of osteoclastogenesis and of the expression of MMPs. The reverse effect is exerted by the Th2-type cytokines such as IL-4 and IL-10 (16, 19). In periodontal lesions, the balance between the expression of Th1 and Th2-type mediators is thought to be a relevant factor in the outcome of disease, possibly regulating the balance of MMPs/TIMPs and RANKL/OPG (12–14, 42, 48).

In the present study, we investigated the pattern of expression of mRNAs encoding for MMPs, TIMPs, RANKL, and OPG in periodontal tissues of *Actinobacillus actinomycetemcomitans*-infected mice, and further correlated them with the profile of cytokines produced and with the patterns of leukocyte migration and alveolar bone loss in the course of experimental periodontal disease.

Material and methods

Induction of periodontal disease

Experimental animal groups comprised 8-week-old male C57BL/6 mice, bred and maintained in the animal house of the Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto-USP. *A. actinomycetemcomitans* (ATCC 29522) was anaerobically grown in supplemented agar medium (TSBV) as previously described (3). Periodontal disease was induced as previously described (11). Briefly, the infection protocol comprised the oral inoculation of 1×10^9 colony forming units of a diluted culture in 100 μl of phosphate-buffered saline with 2%; carboxymethylcellulose using a micropipette. This procedure was repeated after 48 and 96 h. Negative controls included sham-infected mice, which received PBS with carboxymethylcellulose solution without *A. actinomycetemcomitans*, and noninfected animals. The experimental protocol was approved by the local Institutional Animal Care and Use Committee.

Quantification of inflammatory cells from periodontal tissues

The inflammatory cells in periodontal tissues were quantified as previously described (11). The whole buccal and palatal periodontal tissues (between the mesial first molar to the distal site of the 3rd molar) of maxillary molars were collected and incubated for 1 h at 37°C, dermal side down on RPMI 1640, supplemented with $NaHCO_3$, penicillin/streptomycin/gentamicin, and 0.28 Wunsch units/ml of liberase blendzyme CI (F. Roche, Hoffmann-La

Roche Ltd, Basel, Switzerland). The tissues of five mice at each time point per group were processed in the presence of 0.05% DNase (Sigma-Aldrich, Steinhein, Germany) using Medimachine (BD Biosciences PharMingen, San Diego, CA) according to the manufacturer's instructions. After processing, cell viability was assessed by Trypan blue exclusion, and the cell count was performed in a Neubauer chamber. Five animals were analyzed at each time point, and the results represent the number of cells (± SD) in the periodontal tissues of each mouse, from one experiment that was representative of three independent experiments.

Quantification of alveolar bone loss

The extent of alveolar bone loss was evaluated as previously described (11). First, the maxillas were hemisected, exposed overnight in 3% hydrogen peroxide, and mechanically defleshed. The palatal faces of the molars were photographed at 20× magnification using a dissecting microscope (Leica, Wetzlar, Germany), with the occlusal face of the molars positioned perpendicular to the base. The images were digitized, and analyzed by a blinded investigator using IMAGETOOL 2.0 software (The University of Texas Health Science Center, San Antonio, TX). The quantification comprises the measurement of the area between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC), in arbitrary units of area (AUA), corresponding to the pixels contained in the selected area in the three posterior teeth. Five animals were analyzed at each time point, and for each animal the alveolar bone loss was defined as the mean of CEJ–ABC between the right and the left arch. The results represent the increase in CEJ–ABC area (± SD) of each mouse in relation to the noninfected controls, for one experiment representative of three independent experiments.

RNA and DNA extraction

The extraction of total RNA of periodontal tissues (palatal and buccal gingival tissue between the mesial maxillary first molar and the distal site of the 3rd molar, and the corresponding alveolar bone of the maxillary molars) was performed with Trizol reagent following the protocol recommended by the manufacturer (Life Technologies, Rockville, MD). Briefly, Trizol (1 ml for 1 mg of tissue) was added to the sample, shaken for 30 s, and incubated at room temperature for 5 min. For each 1 ml of the suspension, 0.2 ml chloroform (Sigma Co., St. Louis, MO) was added and centrifuged at 12,000 *g* for 15 min at 4°C. The aqueous phase was transferred to a new tube, to which the same volume of isopropanol was also added. The sample was shaken, incubated for 20 min at 20°C and centrifuged again as previously described. The precipitate was washed in 100% ethanol and dried at room temperature. RNA samples were suspended in 50 µl of deionized RNAse-free water and stored at − 70°C. An aliquot of 5 µl was used to obtain the concentration of RNA/µl in the samples, using the GeneQuant method (Pharmacia Amersham Biosciences, Piscataway, NJ). DNA extraction for the quantification of *A. actinomycetemcomitans* detection was performed from periodontal tissue samples homogenized in sterile Milli-Q water, from which DNA extraction was performed with the DNA Purification System, following the protocol recommended by the manufacturer (Promega Biosciences Inc, San Luis Obispo, CA).

RealTime polymerase chain reactions (PCR)

To quantify *A. actinomycetemcomitans,* RealTime PCR was performed as previously described (38), using 5 ng DNA. For the mRNA quantification, complementary DNA (cDNA) was synthesized using 3 µg RNA through a reverse transcription reaction (Superscript II, Gibco Life Technologies, Grand Island, NY). RealTime PCR quantitative mRNA or DNA analyses were performed in an ABI Prism 7000 Sequence Detection System using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK) to quantify amplicons. The standard PCR conditions were 95°C for 10 min, then 40 cycles at 94°C (1 min), 56°C (1 min), and 72°C (2 min), followed by the standard denaturation curve. The sequences of primers were designed using PRIMEREXPRESS software (Applied Biosystems) using nucleotide sequences present in the GenBank database. The primer sequences, predicted amplicon sizes, annealing and melting temperatures are presented in Table 1. PCR conditions for each target were conscientiously optimized with regard to primer concentration, absence of primer-dimer formation, and efficiency of amplification of target genes and housekeeping gene control. SYBR Green PCR Master Mix (Applied Biosystems), 200 nM specific primers, and 2.5 ng cDNA (or 5 ng DNA) were used in each reaction. The threshold for positivity of RealTime

Table 1. Primer sequences and reaction properties

Target	Sense and antisense sequences	tA (°C)	tM (°C)	bp
MMP-1	TGGACCTGGAGGAAATCTTGC AGAGTCCAAGAGAATGGCCGA	58	79	155
MMP-2	CTGATGGCACCCATTTACACCT GATCTGAGCGATGCCATCAAA	60	82	186
MMP-9	AGAGATGCGTGGAGAGTCGAA AAGGTTTGGAATCTGCCCAGG	65	85	162
TIMP-1	ACTGCAGGATGGACTCTTGCA TTTCAGAGCCTTGGAGGAGCT	30	82	206
TIMP-2	CAAGTTCTTCGCCTGCATCAA TCGAAACCCTTGGAGGCTT	61	84	155
TIMP-3	TTCTCAGCGAGGATGGCACTT AAACACGGTTCAGGATGCTGG	60	81	200
RANKL	CAGAAGATGGCACTCACTGCA CACCATCGCTTTCTCTGCTCT	65	73	203
OPG	GGAACCCCAGAGCGAAATACA CCTGAAGAATGCCTCCTCACA	57	77	225
Cathepsin K	CTCCCTCTCGATCCTACAGTAATGA TCAGAGTCAATGCCTCCGTTC	58	79	306
TNF-α	AAGCCTGTAGCCCATGTTGT CAGATAGATGGGCTCATACC	56	79	330
IFN-γ	ATGAAATATACAAGTTATATCATG TGTTTCGAGGTCGAAGAGCATCCC	58	77	501
IL-12	AGCACCAGCTTCTTCATCAGG GCGCTGGATTCGAACAAAG	58	76	164
IL-4	GCGATA TCACCTTACA GGAG TGTCCTGTG AAGGAAGCCAAC	58	82	308
IL-10	AGATC TCCGAGATGC CTTCA CCGTGGAGCAGGTGAAGAAT	58	85	307
β-actin	ATGTTTGAGACCTTCAACA CACGTCAGACTTCATGATGG	56	75	495
A. actinomycetemcomitans	ATGCCAACTTGACGTTAAAT AAACCCATCTCTGAGTTCTTCTTC	60	78	557

At, annealing temperature. Mt, Melting temperature. bp, base pairs of amplicon size.

PCR was determined based on negative controls. For mRNA analysis the calculations for determining the relative level of gene expression were made according to the instructions from the User's Bulletin (P/N 4303859) from Applied Biosystems, by reference to the beta-actin in the sample, using the cycle threshold (Ct) method. The mean Ct values from duplicate measurements were used to calculate the expression of the target gene with normalization to a housekeeping gene used as internal control (beta-actin), using the 2–ÄCt formula, also according to the User's Bulletin. For DNA analysis, the calculations for determining the level of gene expression were also made using the cycle threshold (Ct) method, and normalized by the tissue weight. The levels of negative controls without RNA or DNA and without reverse transcriptase were also calculated. One experiment representative of three is presented in the Results.

Protein extraction and cytokine ELISA

Measurements of cytokines in periodontal tissues were performed as previously described (41). For protein extraction, palatal periodontal tissue was homogenized in phosphate-buffered saline (PBS) pH 7.4, centrifuged at 1000 r.p.m. at 4°C and the supernatants were stored at − 70°C. The concentrations of cytokines in periodontal extracts were determined by ELISA using commercially available kits (all from R & D Systems, Minneapolis, MN) as follows: IL-1β (sensitivity > 3 pg/ml), TNF-α (> 3.4 pg/ml), IFN-γ (> 2 pg/ml), IL-4 (> 2 pg/ml), and IL-10 (> 4 pg/ml). All assays were carried out according to the manufacturer's instructions. The results were expressed as picograms of cytokine (± SD) per milligram of periodontal tissue, for one experiment representative of three.

Statistical analysis

Inflammatory cells counts and CEJ-ABC area values were submitted to the statistical test One-Way ANOVA, followed by Bonferroni's post test. To assess possible differences in the intensity of mRNA expression and in the cytokine levels in the course of disease, ANOVA was performed, followed by Bonferroni's test. Linear regression analysis was used to test possible correlations between the levels of expression of MMPs, TIMPs, RANKL and OPG compared to levels of cytokines expression. For all the tests used, values of $P < 0.05$ were considered statistically significant. All statistical tests were performed with GRAPHPAD INSTAT 3.05 and GRAPHPAD PRISM 3.0 software (GraphPad Software Inc, San Diego, CA).

Results

Analysis of the inflammatory reaction, alveolar bone loss and periodontal infection

In order to confirm the establishment of experimental periodontal disease we evaluated key events of the disease, the inflammatory reaction and alveolar bone loss. We found that *A. actinomycetemcomitans*-infected mice presented a significant increase in the number of leukocytes extracted from periodontal tissues at 7, 15, 30 and 60 days ($P < 0.001$) post infection, when compared with sham-infected and noninfected mice (Fig. 1A). Histological analysis has demonstrated that there is a marked leukocyte infiltration in connective periodontal tissue of *A. actinomycetemcomitans*-infected mice, supporting the hypothesis that cells extracted from this tissue are representative of inflammatory migrating cells. We also verified that *A. actinomycetemcomitans*-infected mice presented progressive alveolar bone loss when compared to sham-infected mice, as shown by the increase in the area between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC) at 15, 30, 45 and 60 days ($P < 0.001$) post infection (Fig. 1B). In addition, we analyzed these data regarding the rate of increase in inflammatory cell counts and alveolar bone loss in two distinct periods during the course of the disease (the initial period, lasting from infection time until 30d after, and the late period, between 30 and 60 days post infection). We found that both inflammatory reaction and alveolar bone loss presented high rates of increase in the initial period (0–30 days). By contrast, in the late period (30–60 days) the increase in both inflammatory ($P < 0.001$) and bone loss ($P < 0.01$) indexes was significantly lower than in the initial period. Regarding periodontal infection, the levels of *A. actinomycetemcomitans*, quantified by RealTime PCR (Fig. 1C), increased until day 7 of infection, and remained constant throughout the course of the experimental disease.

Quantitative analysis of MMP and TIMP mRNA expression

To evaluate the role that the balance between MMPs and TIMPs plays in the

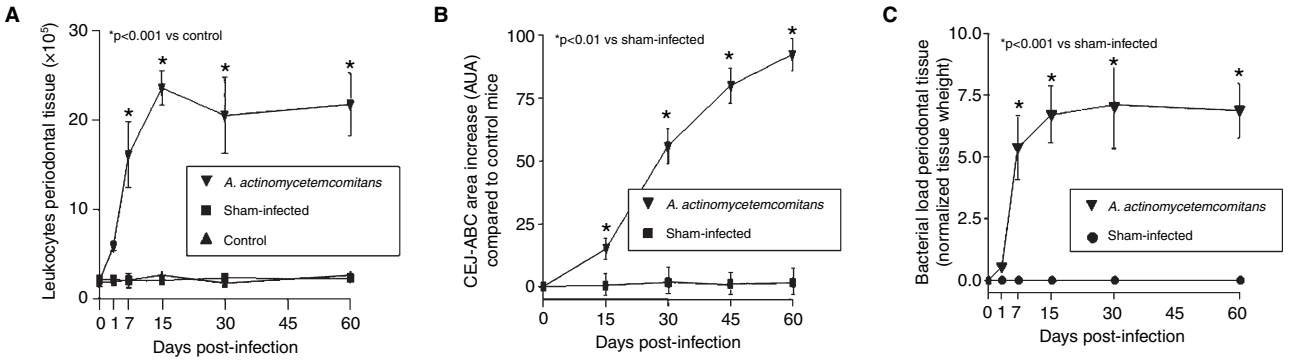

Fig. 1. Inflammatory reaction, alveolar bone loss, and periodontal infection after oral inoculation of *A. actinomycetemcomitans* in mice. After inoculation of C57BL/6 mice, periodontal samples were taken and evaluated for: A) total count of leukocytes extracted from periodontal tissues; B) alveolar bone loss, determined through the measurements of CEJ-ABC area in the palatal face of maxillary molars, with IMAGETOLL2.0 software. A: *****$P < 0.001$; B: *****$P < 0.01$; One-way ANOVA, Bonferroni's post test. C) Levels of *A. actinomycetemcomitans* infection in periodontal tissues were assessed by RealTime PCR, as described in Material and methods. *****$P < 0.001$; One-way ANOVA, Bonferroni's post test. Values (mean +SD) obtained from five animals at each time point, from one experiment representative of three. Statistical tests performed with GRAPHPAD PRISM 3.0 software (GraphPad Software Inc.).

course of experimental periodontal disease, we first determined the kinetics of their mRNA expression in periodontal tissues from *A. actinomycetemcomitans*-infected C57BL/6 mice (Fig. 2).

The expression of MMPs (MMP-1, MMP-2, MMP-9) and TIMPs (TIMP-1, TIMP-2 and TIMP-3) was found to be constitutive, with low levels being detected at 0 h (Fig. 2). During the course of the disease, the expression of MMP-1 was found to be increased at 24 h, 7 and 15 days post infection, but there was a significant decrease at 30 and 60 days post infection (Fig. 2). Similarly, the expression of MMP-2 and MMP-9 showed a progressive increase from 24 h until 15 days of infection, with a decrease at 30 and 60 days (Fig. 2). In contrast, there was a modest increase in the expression of TIMPs (TIMP-1, TIMP-2 and TIMP-3) after 24 h until 15 days of infection, and a significantly augmented expression at 30 and 60 days (Fig. 2).

The levels of expression of the housekeeping gene β-actin were used as positive controls and to normalize the levels of the other target genes studied. The results show distinct patterns of MMP/TIMP expression in the course of experimental disease; one early phase with high MMP and low TIMP expression, followed by a late phase, after 30 days of infection, with lower MMP and higher TIMP expression.

Quantitative analysis of osteoclast factors mRNA expression

We next investigated the expression of the osteoclastogenic factor RANKL, its antagonist OPG, and cathepsin K, a key enzyme

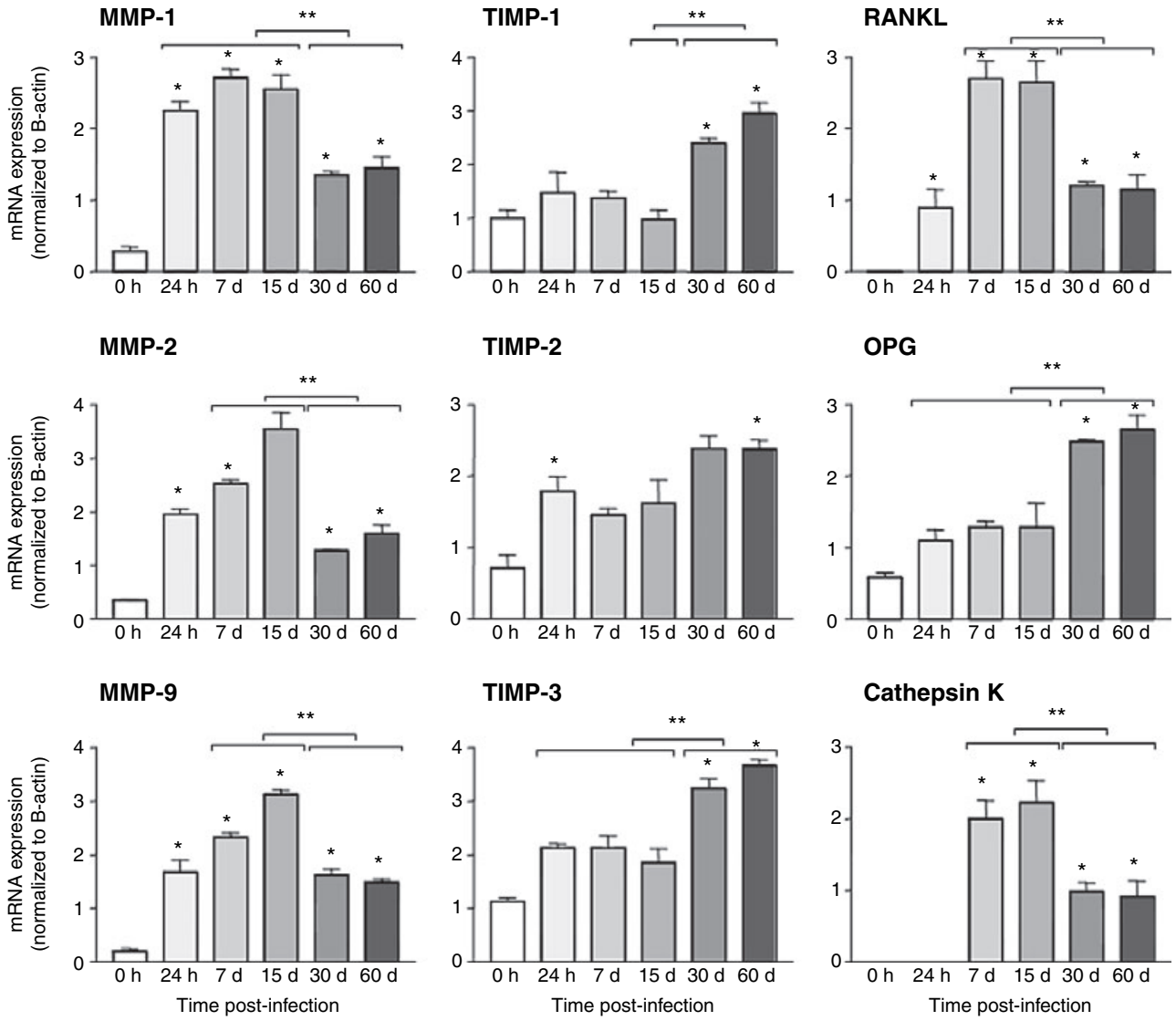

Fig. 2. Kinetics of MMPs, TIMPs, RANKL and OPG in the course of experimental periodontal disease. Periodontal tissues of C57BL/6 mice inoculated orally with *A. actinomycetemcomitans* were harvested from zero (before infection) until 60 days of infection. The levels of MMPs, TIMPs, RANKL, OPG, and cathepsin K mRNA were quantified by RealTime PCR SYBR-Green System, as described in Material and methods. The results are presented as the expression of the individual mRNAs with normalization to β-actin, using the Ct method. The results (mean +SD) represent values from duplicate measurements from one experiment representative of three. ***P* < 0.05 compared to 0 h, ****P* < 0.05 compared to selected bars, One-Way ANOVA followed by Bonferroni's post test, performed with GRAPHPAD PRISM 3.0 software (GraphPad Software Inc.).

in the bone resorption process (Fig. 2). The expression of RANKL was detected at 24 h after infection, and at 7 and 15 days of infection its expression was significantly increased, with a significant decrease being found at 30 and 60 days. Cathepsin K was highly expressed at 7 and 15 days of infection but decreased significantly at 30 and 60 days post infection. In contrast, the expression of OPG presented a small increase at 24 h, 7 and 15 days after infection, while a significant increase was found at 30 and 60 days post infection. Similar to what was seen with MMPs/TIMPs, an initial high expression of RANKL and cathepsin K was followed by a significant decrease in their messages and a simultaneous increase in OPG expression.

Quantitative analysis of cytokines mRNA expression

We next asked whether the differences in the expression of MMPs, TIMPs, RANKL, and OPG could be due to the differential expression of cytokines that are known to regulate their expression (Fig. 3). Infected mice exhibited high levels of messages for TNF-α, IL-1β, and IFN-γ at 24 h, 7d and 15 days after infection, and this expression was found to be decreased at 30 and 60 days (Fig. 3). The expression of IL-12 was found to be higher at 24 h after infection, and then decreased after 7 days of infection. The expression of IL-10 message was detected at low levels on days 7 and 15, and then presented a significant increase at 30 and 60 days post infection. IL-4 mRNA was only detected at 30 and 60 days at similar levels. Control groups showed no cytokine mRNA expression in periodontal tissues (data not shown). The results show that cytokines produced by both T helper subsets are expressed in the course of experimental disease, with an initial predominance of Th1-mediator expression followed by a rise in Th2-type cytokine expression after 30 days post infection.

ELISA analysis of cytokines in periodontal tissues

To confirm RealTime PCR findings, we also performed ELISA for the quantification of levels of cytokine protein in the course of experimental periodontal disease. As shown in Fig. 3, infected mice exhibited high levels of TNF-α, IL-1β, and IFN-γ in periodontal tissues at 24 h, 7 and 15 days post infection, and such production was found to be decreased at 30 and 60 days after infection (Fig. 3). IL-4 and IL-10 proteins were only detected at 30 and 60 days post infection. Control groups showed no cytokine production in periodontal tissues (data not shown).

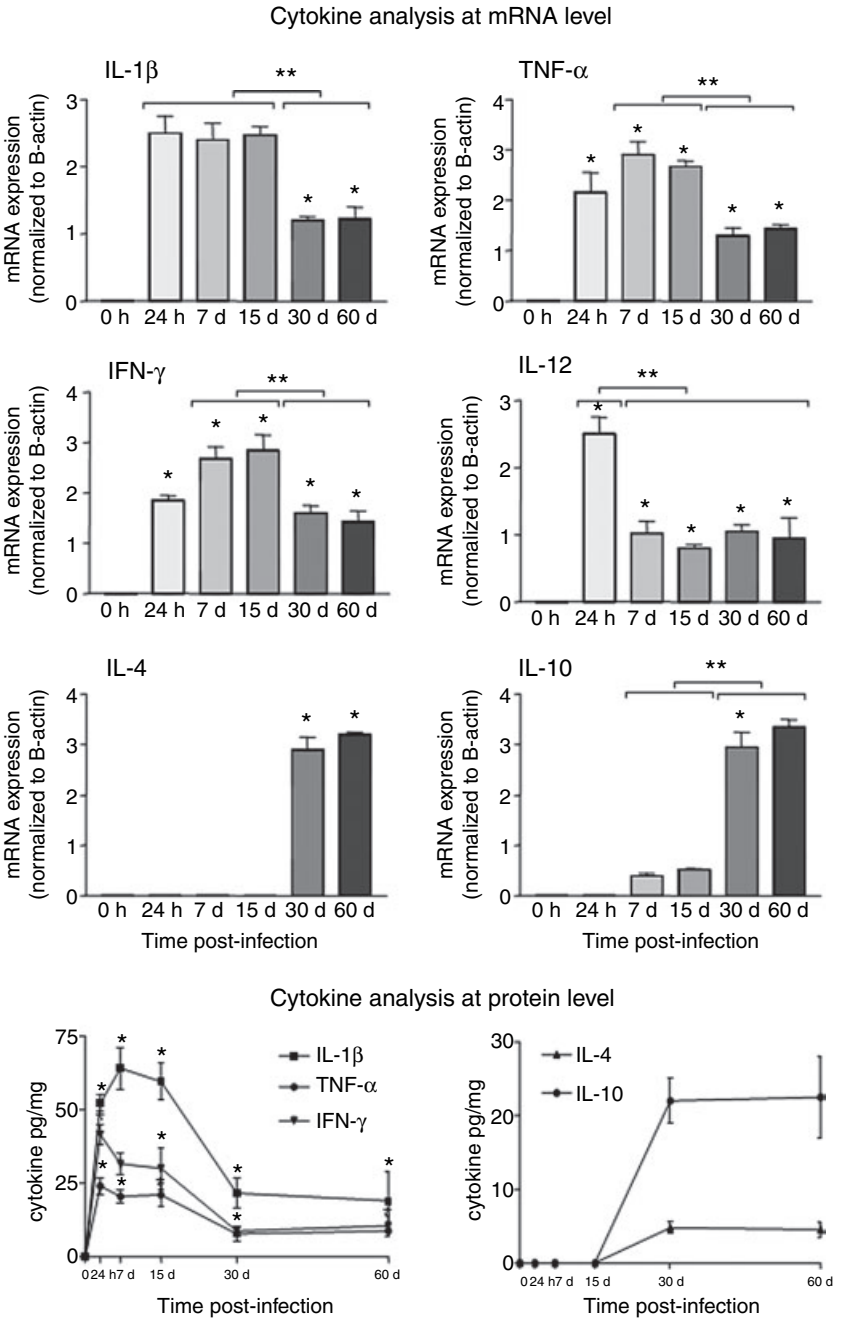

Fig. 3. Kinetics of cytokine production in the course of experimental periodontal disease. Periodontal tissues of C57BL/6 mice inoculated orally with *A. actinomycetemcomitans* were harvested from zero (before infection) until 60 days of infection. Cytokine production were analyzed at both mRNA and protein levels. The levels of IL-1β, TNF-α, IFN-γ, IL-12, IL-10, and IL-4 mRNA were quantified by RealTime PCR SYBR-Green System, as described in Material and methods. The results are presented as the expression of the individual mRNAs with normalization to β-actin from duplicate measurements from one experiment representative of three. The levels of IL-1β, TNF-α, IFN-γ, IL-10, and IL-4 protein in periodontal tissues were determined at the indicated times by ELISA, as described in Material and methods. The results are presented as picograms of cytokine per milligram of tissue, mean ± SD from duplicate measurements, one experiment representative of three. $*P < 0.05$ compared to 0 h, $**P < 0.05$ compared to selected bars, One-Way ANOVA followed by Bonferroni's post test, performed with GRAPHPAD PRISM 3.0 software (GraphPad Software Inc.).

Correlation analysis of the expression of MMP, TIMPs, RANKL and OPG with expression of cytokines in diseased periodontal tissues

We also used linear regression analysis to investigate the possible correlations between the levels of expression of MMPs, TIMPs, RANKL, and OPG and the levels of cytokines expressed in periodontal tissues. We found positive correlations between the levels of IFN-γ, TNF-α, and IL-1-β expression with both MMP-1 and RANKL, suggesting that these potential mediators of tissue destruction are induced by inflammatory and Th1-type cytokines (Table 2). Positive correlations were also found between IFN-γ and MMP-2, MMP-9 and cathepsin K; TNF-α with MMP-2 and MMP-9; IL-1-β and MMP-2. We also found positive correlations of IL-4 and IL-10 with OPG and TIMP-1, IL-4 and TIMP-3; and IL-10 with both TIMP-2 and TIMP-3 (Table 2), suggesting that these Th2-type cytokines modulate their expression in the course of experimental periodontal disease.

Discussion

Oral inoculation of the widely studied putative human periodontopathogen *A. actinomycetemcomitans* in mice leads to the development of periodontal disease, characterized by an intense and widespread migration of leukocytes to the gingival tissues and marked alveolar bone resorption (11). We used such an experimental model to investigate the expression of the potentially tissue destructive mediators MMP and RANKL, and their respective inhibitors TIMP and OPG in the periodontal tissues of *A. actinomycetemcomitans*-infected mice.

The data presented here show that MMPs and TIMPs, which are constitutively expressed at low levels in healthy periodontal tissues, increased significantly after *A. actinomycetemcomitans* infection of mice. Similarly, in humans, the expression of MMPs and TIMPs is regularly found in healthy periodontal tissues, and they are supposed to be involved in the physiological turnover of periodontal tissues (9, 17). Interestingly, we found two apparently distinct phases of MMP/TIMP expression in the course of experimental periodontal disease, associated with time periods with remarkable differences in the rate of disease progression. In the early stage of disease, from 24 h until 30 days of infection, there was a marked increase in both inflammatory cell number and bone loss and a significant increase in MMP expression. After 30 days of infection, in a late disease period, a stable number of inflammatory cells in periodontal tissue were found together with a lower rate of alveolar bone loss, a decreased expression of MMPs and an increased expression of TIMPs. These data suggest that the balance between MMPs and TIMPs expression could account for the different degrees of disease progression. In agreement with this, the increased expression of MMPs in diseased periodontal tissues accounts for the destruction of connective and bone tissues (1, 6, 18, 31). Furthermore, differential expression of TIMPs was reported in aggressive and chronic human periodontitis, which could account for the degree of disease severity (13). However, data regarding the expression of TIMPs in healthy vs. diseased human periodontal tissues are contradictory. Some studies show a decrease in the levels of TIMPs in diseased periodontal tissues, supporting the idea that an imbalance in the levels of TIMPs/MMPs occurs in periodontal diseases and results in tissue destruction (40, 47). Conversely, other studies detected an increased expression of TIMPs in diseased periodontal tissues (2, 13, 17, 30), which could reflect an attempt to maintain the tissue homeostasis, in view of the increased expression of MMPs. However, such up-regulation of TIMPs may not be enough to compensate for the even higher up-regulation of MMPs, and such an imbalance may result in periodontal destruction. Nevertheless, imbalances in the MMP/TIMP system (i.e. lower levels of TIMPs and/or higher levels of MMPs) are involved in the pathogenesis of several diseases including rheumatoid arthritis (20, 25, 37, 39, 51), which share several features with periodontal diseases, including the chronic nature of the inflammatory reaction and tissue destruction (28).

Since the alveolar bone resorption is another key feature of periodontal diseases, we also investigated the expression of factors involved in osteoclast differentiation and activation (43). In a similar pattern to that seen with MMPs/TIMPs, we found that the early phase of experimental periodontitis (0–30 days) was associated

Table 2. Correlations between the levels of expression of mRNA for MMPs, TIMPs, RANKL and OPG and mRNA for cytokines in the course of experimental periodontal disease

	IL-1β	TNF-α	IFN-γ	IL-4	IL-10
MMP-1	0.8040 ± 0.1066 0.9343	0.8582 ± 0.04705 0.9881	0.7808 ± 0.1347 0.8935		
MMP-2	0.8994 ± 0.2112 0.8193	0.9803 ± 0.1602 0.9035	0.9005 ± 0.2017 0.8329		
MMP-9		0.8947 ± 0.2275 0.7945	0.8759 ± 0.1968 0.8319		
TIMP-1				0.4415 ± 0.08603 0.8682	0.4725 ± 0.1001 0.8479
TIMP-2					0.2936 ± 0.09630 0.6991
TIMP-3				0.5701 ± 0.1053 0.8800	0.6153 ± 0.1168 0.8741
RANKL	0.7378 ± 0.2376 0.7068	0.8770 ± 0.1229 0.9271	0.8373 ± 0.1208 0.9232		
OPG				0.5458 ± 0.09091 0.9001	0.6011 ± 0.08193 0.9308
Cathepsin K			0.8724 ± 0.2854 0.7002		

Linear regression analysis was used to test the correlations between the levels of expression of MMPs, TIMPs, RANKL, OPG and cathepsin K and the levels of the cytokines IL-$^{1\beta}$, TNF-α, IFN-γ, IL-4 and IL-10 in the course of experimental periodontal disease. Total RNA was extracted, and the individual levels of mRNA were quantified by a RealTime PCR SYBR-Green System, as described in Material and methods. Linear regression was performed with the GRAPHPAD PRISM 3.0 software (GraphPad Software Inc.). Statistically significant values of slope and r^2 are identified in the Table.

with high cathepsin K and RANKL expression and low OPG expression. By contrast, in the late period of experimental periodontal disease (after 30 days), characterized by the lower disease activity, lower levels of cathepsin K and RANKL and higher levels of OPG expression were detected. This suggests that the RANKL/OPG balance may determine the rate of alveolar bone loss and, consequently, the progression of experimental disease. Such a hypothesis is supported by studies showing that RANKL is closely associated with bone resorption (34, 43), and that the blockade of RANKL by OPG leads to a reduction in the alveolar bone loss (45). Furthermore, the modulation of the RANKL/OPG system is supposed to lead to modulation of human periodontal diseases severity (8, 13, 29).

We next investigated the cytokines potentially involved in the modulation of MMP/TIMP and RANKL/OPG balance (5, 6, 34, 43). We found high levels of TNF-α, IL-1β, and IFN-γ in periodontal tissues during the early phase of experimental periodontal disease, which were significantly decreased after 30 days of infection. Furthermore, there were positive correlations in the expression of MMPs, RANKL, and cathepsin K with TNF-α, IL-1β, and IFN-γ, suggesting that such inflammatory and Th1-type cytokines play a detrimental role in the pathogenesis of periodontal diseases. In agreement with our data, previous studies demonstrated that IL-1 and TNF-α clearly trigger deleterious events occurring in periodontal disease, including the stimulation of matrix metalloproteinase production and bone resorption (6, 16, 43). Moreover, IFN-γ appears to be the predominant cytokine produced by T cells in periodontal lesions, and an increase in IFN-γ-producing cells in the lesions was correlated with the progression of disease (21, 35, 48).

Conversely, the levels of IL-4 and IL-10 increased significantly after 30 days of infection, and were correlated with the higher expression of TIMPs and OPG. The late phase of experimental periodontal disease thus seems to be modulated by IL-4 and/or IL-10 up-regulation of TIMP and OPG expression. In agreement with this, Th2 cytokines (IL-4 and IL-10) are widely expressed in diseased periodontal tissues and are associated with attenuation of disease severity (10, 22, 26, 46). Moreover, despite controversies regarding the role of T-helper cytokines in periodontal diseases, Th1 and Th2 cytokines have been shown to be associated with activation and suppression of bone resorption, respectively (10, 21, 22). In addition, recent studies have demonstrated that mixed patterns of immune response are present in diseased periodontal tissues (11, 12, 44, 48), suggesting that the balance of T-helper responses could account for the control of disease severity through the regulation of MMP/TIMP and RANKL/OPG systems (13, 29, 49).

Our results suggest that pro- and anti-inflammatory cytokines could play distinct roles in the regulation of the balance of MMPs/TIMPs and the RANKL/OPG, which in turn modulates the progression of the *A. actinomycetemcomitans*-induced periodontal disease in mice. However, although the inoculation of viable *A. actinomycetemcomitans* resulted in the induction of periodontal disease in mice, which resembles human periodontitis in several aspects, there are limitations in the interpretation of our findings, as only a single bacterial strain was studied. Further investigation is required to extend this model to include different species or strains of periodontopathogens, nonviable controls, and nonpathogenic bacteria.

Knowledge regarding the role of the cytokines in the outcome of periodontal diseases may provide the basis for future therapeutic interventions aimed at limiting the inflammatory process and tissue damage while improving the repair of periodontal tissues.

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