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Original article

# *Actinobacillus actinomycetemcomitans*-induced periodontal disease in mice: patterns of cytokine, chemokine, and chemokine receptor expression and leukocyte migration

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**Abstract**

Although the pathogenesis of periodontal disease (PD) is not well known, cytokines, chemotactic factors and inflammatory cells are certainly involved in the disease outcome. Here, we characterized the evolution of the PD induced by *Actinobacillus actinomycetemcomitans* in mice, showing that oral inoculation of these bacteria leads to the migration of leukocytes to periodontal tissues and marked alveolar bone resorption. We found the expression of pro-inflammatory and Th1-type cytokines including TNF-α, IFN-γ and IL-12 in periodontal tissues after infection with *A. actinomycetemcomitans*, from the early stages after infection and throughout the course of the disease. Similar kinetics of expression were found for the chemokines CCL5, CCL4, CCL3 and CXCL10 and for the receptors CCR5 and CXCR3, all of them linked to the Th1-type pattern. The expression of the Th2-type mediators IL-10, CCL1 and their receptors CCR4 and CCR8 was detected only after 30 days of infection, determining a time-dependent mixed pattern of polarized immune response. The chemokine expression was correlated with the presence of polymorphonuclear leukocytes, macrophages, CD4 and CD8 lymphocytes, and B cells in the inflammatory infiltrate. Interestingly, during the predominance of the Th1-type response, a sharp increase in the number of inflammatory cells and intense bone loss was seen. By contrast, after the increased expression of Th2-type mediators, the number of inflammatory cells remained constant. Our data demonstrate that mice subjected to oral inoculation of *A. actinomycetemcomitans* represent a useful model for the study of PD. In addition, our results suggest that expression of cytokines and chemokines can drive the selective recruitment of leukocyte subsets to periodontal tissues, which could determine the stable or progressive nature of the lesion.


*Keywords:* Experimental periodontal disease; *Actinobacillus actinomycetemcomitans*; Cytokines; Chemokines; Chemokine receptors; Cell migration; Inflammation

## 1. Introduction

Periodontal disease (PD), a chronic inflammatory disease of the attachment structures of the teeth, is 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 surface of the tooth in close association with periodontal tissues, is the etiologic factor of this disease. The biofilm hosts some typical periodontopathogens, such as *Actinobacillus actinomycetemcomitans*, one of the main etiological agents of localized aggressive human periodontitis and of a large number of cases of chronic periodontitis [1]. The virulence factors of this pathogen include leucotoxins, immunosuppressive factors, and a high capacity to invade the host cells [2–4]. In vitro, *A. actinomycetemcomitans* induces the expression of several cytokines and chemok-

*Abbreviations:* PD, periodontal disease; pi, post infection; PMN, polymorphonuclear leukocyte; RPA, RNAse protection assay; Th, T helper lymphocyte.

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ines by different cell types [5,6], which are possibly involved in mediating the migration of leukocytes to the affected tissue.

Indeed, the development of PD seems to be related to the extension of the inflammatory cell infiltrate to the deeper periodontal tissues [7]. Polymorphonuclear leukocytes (PMNs), the first cell type found in PD lesions, appear to provide the primary immune protection [8,9]. When the aggressive stimulus is not eliminated, it may lead to chronic PD, characterized by a cellular infiltrate dominated by mononuclear cells. Macrophages are outnumbered in chronic inflammatory lesions of PD and are thought to participate in the local immune response through presentation of antigen, killing of pathogens and production of inflammatory mediators [10,11]. Regarding lymphocytes, whereas T lymphocytes predominate in the established chronic lesion, the proportion of B cells and plasma cells increases with the progression of the disease [11–13].

This host response to periodontopathogens protects against the infection, but the persistence of pathogens and the exacerbated immune response may render the protective roles of inflammatory cells dangerous to the host tissues. Inasmuch as the chemotactic factors produced in the lesions certainly are involved in the pathogenesis of PD, their identification is fundamental to guide the development of strategies for controlling the disease.

Chemokines, a family of chemotactic cytokines, attract leukocyte populations by means of their interaction with specific receptors that are members of the seven-transmembrane spanning G protein-coupled receptor family, selectively expressed in these cells [14]. Besides their chemoattractant activity, chemokines also are implicated in the polarization of the immune response, in leukocyte activation, and in the pathogenesis of several diseases [15,16]. Some chemokines have been found in diseased human periodontal tissues [17–20], but their role in the pathogenesis of PD remains unknown. We detected Th1-type chemokines and chemokine receptors in gingival biopsies of patients with aggressive periodontitis, and their expression may favor the migration of the IFN-γ-producing cells found in the lesions. Conversely, in chronic periodontitis there was predominance in the expression of Th2 chemokines, which correlated with the higher expression of IL-10 [20]. Therefore, the selective chemoattraction of T helper (Th) subsets could influence the clinical outcome of PD. In fact, the polarization of immune responses can determine the prognosis of several diseases [21–23], such as arthritis, that share several features with PD, such as the chronic nature of the inflammatory reaction with concomitant bone resorption [24].

The role of chemokines and cytokines in the orchestration of cellular traffic to periodontal tissues is not known, and data are lacking regarding the relevance and kinetics of their expression in the course of human PD. In addition, variables such as the different compositions of periodontal biofilm, the age at onset of disease and the genetic variability of hosts may hinder the interpretation of human studies. Consequently, experimental mouse models are useful to study PD, since they present several advantages including multiple strains with known genetic background, ease of handling, availability of experimental reagents, and the susceptibility to induction of PD [25–27].

Therefore, our aim was to characterize a mouse model of PD induced by *A. actinomycetemcomitans*. We investigated the expression patterns of chemokine, chemokine receptor and cytokine mRNA, and the kinetics of cell migration and alveolar bone loss throughout the course of experimental PD. We conclude that this murine model could be useful for testing hypotheses relevant to human PD.

## 2. Materials and methods

### 2.1. Mice

Experimental groups comprised 8-week-old male C57BL/6 mice, bred and maintained in the animal facilities of the Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto-USP. Throughout the period of the study mice were fed with sterile standard solid mice chow (Nuvital, Curitiba, PR), and sterile water. Mice colonies were free from the following periodontopathogens: *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Eikenella corrodens*. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use.

### 2.2. Induction of PD/periodontal infection

*A. actinomycetemcomitans* (ATCC 29522) was grown anaerobically in supplemented agar medium (TSBV) as previously described [28]. Initially the animals received a direct injection of $1 \times 10^9$ CFU of a diluted culture in 10 μl of PBS into the palatal gingival tissue of the second molar. Immediately after, $1 \times 10^9$ CFU of a diluted culture in 100 μl of PBS with 2% of carboxymethylcellulose (used to facilitate the retention of the bacterial suspension in the oral cavity) was placed in the oral cavity with a micropipette; and after 48 and 96 h only this procedure was repeated. This protocol represents the combination of the number of bacteria and the number of inoculations effective in promoting the colonization of the oral cavity by *A. actinomycetemcomitans* and the establishment of PD in 100% of the animals. The effectiveness of infection was confirmed by the detection of *A. actinomycetemcomitans* in periodontal tissues by polymerase chain reaction (PCR) (as previously described [28]) at all the times analyzed. Positive controls received a single injection of LPS (5 μg in 10 μl of PBS—*E. coli* LPS—Sigma Co., St. Louis, MO). Negative controls included sham-infected mice, which received PBS with carboxymethylcellulose solution without *A. actinomycetemcomitans*, and non-infected animals.

### 2.3. RNA extraction

Total RNA was extracted from gingival palatal tissues between the mesial of the first molar and the distal site of the

third molar. This was performed with the 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 of chloroform (Sigma Co.) 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 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 per µl in the samples, using the GeneQuant kit (Pharmacia Amersham Biosciences, Piscataway, NJ, USA).

### 2.4. RNAse protection assay (RPA)

Total RNA was extracted as previously described. RPA was performed with multiprobe template mCK-5—containing DNA templates for the chemokines: XCL-1 (lymphotactin/LTN), CCL5 (RANTES), CCL11 (eotaxin), CCL3 (MIP-1α), CCL4 (MIP-1β), CXCL1 (MIP-2), CCL2 (JE), and the housekeeping genes L32 and GAPDH following the manufacturer's protocol (BD Biosciences PharMingen, San Diego, CA). Briefly, the DNA template was used to synthesize the α-[$^{32}$P]UTP (3000 Ci/mmol, 10 mCi/ml, Amersham Life Science, Buckinghamshire, UK) labeled probes in the presence of a GACU pool using a T7 RNA polymerase. Hybridization with 20 µg of each target RNA was performed overnight followed by digestion with RNAse A and T1. The samples were treated with a mixture of proteinase K-SDS and then extracted with chloroform and precipitated by the addition of ammonium acetate. The samples were loaded on an acrylamide-urea sequencing gel next to DNA molecular weight markers and labeled probes, and run at 50 W with Tris–borate/ethylenediaminetetraacetic acid (EDTA) electrophoresis buffer (TBE). The gel was adsorbed onto a filter paper and dried under vacuum. The radioactivity of [α-$^{32}$P]-labeled probes was measured by phosphorimaging (STORM Phosphorimaging, BD Biosciences PharMingen), used to visualize the results. Since only the samples were submitted to proteinase digestion, they are visualized in different positions to the DNA molecular weight markers in the gel. At each time point, two animals per group were individually analyzed.

### 2.5. RT-PCR reactions

The complementary DNA (cDNA) was synthesized by means of a reverse transcription reaction (Superscript II, Gibco-Life Technologies, Carlsbad, CA, USA) using 3 µg of RNA. PCRs were them performed in a final volume of 50 µl containing 2.5 mM $MgCl_2$, 2.5 U of the enzyme Taq polymerase (Gibco-Life Technologies), and specific primers at 1 µM by using the PTC-100 cycler (MJ Research, Watertown, MA). The basic conditions of the reaction were 30 cycles of 1 min at 94 °C for denaturation, 1 min at 54 °C annealing and 2 min at 72 °C of extension, plus a final step of extension for 7 min at 72 °C. The sequences of the primers used are given in Table 1. The detection of mRNA for beta actin was used as a positive control, while a water sample was used as a negative control. The amplification products of PCR were separated by electrophoresis on a 6% acrylamide gel and visualized as bands by 0.2% silver nitrate staining. At each time point, three animals per group were individually analyzed.

### 2.6. Histological analysis

Four animals selected at random from each group were sacrificed at 0, 15, 30, and 60 days after infection. The periodontal tissues obtained were then fixed in 4% paraformaldehyde in PBS at pH 7.4 for 12 h at room temperature. The specimens were demineralized thoroughly in 10% EDTA 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 Co.). Histological assessment was carried out following routine hematoxylin and eosin staining.

Table 1
Primers sequences and reaction properties

| Target | Sense and anti-sense sequences | tA [a] (°C) | bp [b] |
|---|---|---|---|
| IFN-γ | GCT CTG AGA CAA TGA ACG CT/AAA GAG ATA ATC TGG CTC TGC | 60 | 229 |
| IL-12 | CCA CAT TCC TAC TTC TC/GTC TAT TCC GTT GTG TC | 58 | 205 |
| TNF-α | AAG CCT GTA GCC CAT GTT GT/CAG ATA GAT GGG CTC ATA CC | 58 | 255 |
| IL-10 | CAA TAA CTG CAC CCA CTT CCC/GAG AAA TCG ATG ACA GCG CCT | 58 | 310 |
| IL-4 | TAT GCG AAG CAC CTT GGA AGC/CTG ACG GCA CAG AGC TAT TGA | 56 | 251 |
| CXCL3 | CC TTG ACC CTG AAG CTC CCT TGG TTC/CGTGCG TGT TGA CCA TAC AAT ATG | 58 | 521 |
| CXCL10 | CA GCACCATGAA CCCAAGTGC/GGT CTTCTGAAAG GTGACCAGC | 58 | 431 |
| CXCR3 | ATC TACCTATCAG CCAACTACGA/T CAGAGAGCAA ATGTGGATGT | 60 | 433 |
| CCR5 | CTC TTC CTG CTC ACA CTA CCA T/TGT GTA GAA AAT GAG GAC TGC A | 60 | 322 |
| CCR4 | CTT GCACCAAGGA AGGTAT/AG CATAGACAGA TACCTAGG | 60 | 334 |
| CCR8 | TGTCCACGCTGTCTATGCCAT/CAGACCCAAGGCGTTGATTT | 58 | 217 |
| β-Actin | TGG AAT CCT GTG GCA TCC ATG A/TAA AAC GCA GCT CAG TAA CAG T | 60 | 349 |

[a] tA: annealing temperature.
[b] bp: base pairs of amplicon size.

### 2.7. Isolation of inflammatory cells from periodontal tissues and flow cytometric analysis

To isolate and characterize leukocytes present in the lesion site, whole buccal and palatal periodontal tissues of upper molars were collected and incubated for 1 h at 37 °C, dermal side down on RPMI 1640, supplemented with $NaHCO_3$, penicillin/streptomycin/gentamycin, and 0.28 Wunsch units/ml of liberase blendzyme CI (Roche-F. 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) 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. For immunofluorescence staining, after cell count, the cells were filtered through a 50-μm filter and then were washed and stained for 20 min at 4 °C with the optimal dilution of each Ab. For immunostaining, PE- and FITC-conjugated Abs against CD4, CD8, GR1 and F4/80, and respective isotype controls were employed (BD Biosciences PharMingen). Cells were washed again and analyzed by flow cytometry (FACScan™ and CELLQuest™ software; BD Biosciences PharMingen). Results represent the number of cells ( ± S.D.) in the periodontal tissues of each mouse, for three independent experiments.

### 2.8. Quantification of alveolar bone loss

Evaluation of the extent of alveolar bone loss was performed similarly as previously described [25]. The maxillae were hemisected, exposed overnight in 3% hydrogen peroxide and mechanically defleshed. The palatal faces of the molars were photographed with 20× magnification using a dissecting microscope (Leica, Wetzlar, Germany), with the occlusal face of the molars positioned perpendicularly to the base. The images were digitized, and analyzed by an investigator unfamiliar with the project, using ImageTool 2.0 software (The University of Texas Health Science Center, San Antonio). Quantitative analysis comprised the measurement of the area between the cemento-enamel 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. At each time point, five animals were analyzed, and for each animal the alveolar bone loss was defined as the average of CEJ–ABC between the right and the left arch.

### 2.9. Statistical analysis

The inflammatory cells number and the CEJ–ABC area values were submitted to the one-way ANOVA statistical test, followed by Tukey's post test. Differences in the relative intensity of mRNA expression between the groups were analyzed by the Kruskal–Wallis test and Dunn's post test. Values of $P < 0.05$ were considered statistically significant. All statistical tests were performed with the Graph Pad InStat 3.05 and Graph Pad Prism 3.0 software (Graph Pad Software Inc., San Diego, CA).

## 3. Results

### 3.1. Kinetics of the expression of messenger RNA for cytokines as measured by RT-PCR

Since the cytokine pattern in periodontal tissue is supposed to determine the outcome of human PD, we therefore evaluated the kinetics of expression for mRNA for the cytokines TNF-α, IFN-γ, IL-12, IL-10 and IL-4 by RT-PCR (Fig. 1) throughout the course of experimental PD. Infected mice exhibited messages for TNF-α, IFN-γ and IL-12 during all of the period analyzed, and these were detected as early as 6 h pi. IL-10 mRNA was only detected at 30 days post infection and was maintained until 60 days, while message for IL-4 was not detected (Fig. 1A). As a positive control, we injected LPS into gingival tissue and evaluated the expression of message for the same cytokines. LPS induced the expression of TNF-α and IFN-γ at 6, 24, 48 h and 3 days pi, while IL-10 and IL-4 mRNA were not detected at these time points (Fig. 1B). Sham-infected and control groups showed no expression of mRNA for these cytokines. The expression of the housekeeping gene β-actin is also shown (Fig. 1. line 6).

### 3.2. Kinetics of chemokine mRNA expression as measured by RPA

Our next aim was to evaluate the kinetics and the pattern of expression of chemokines in the course of the disease. We performed RPA in order to determine the kinetics of the quantitative expression of mRNAs for the chemokines XCL1,

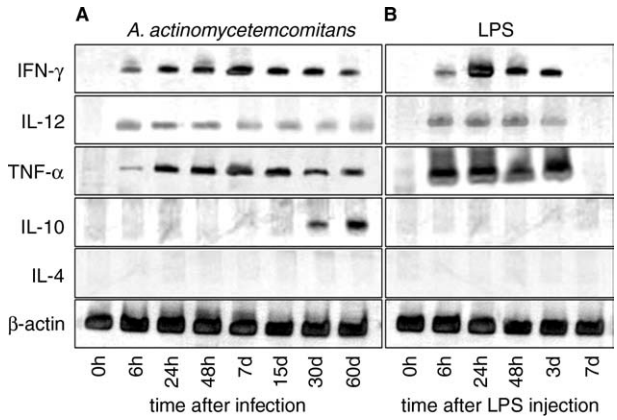


Fig. 1. Kinetics of cytokine mRNA expression in the course of experimental PD. Periodontal tissues of C57BL/6 mice inoculated orally with *A. actinomycetemcomitans* (A) or LPS (B) were harvested from zero (before infection) until 60 days of infection. The RNA was isolated and the transcripts for the cytokines IFN-γ, IL-12, TNF-α, IL-10, and IL-4 were detected by RT-PCR, as described in Section 2. The results shown are representative of three independent experiments.

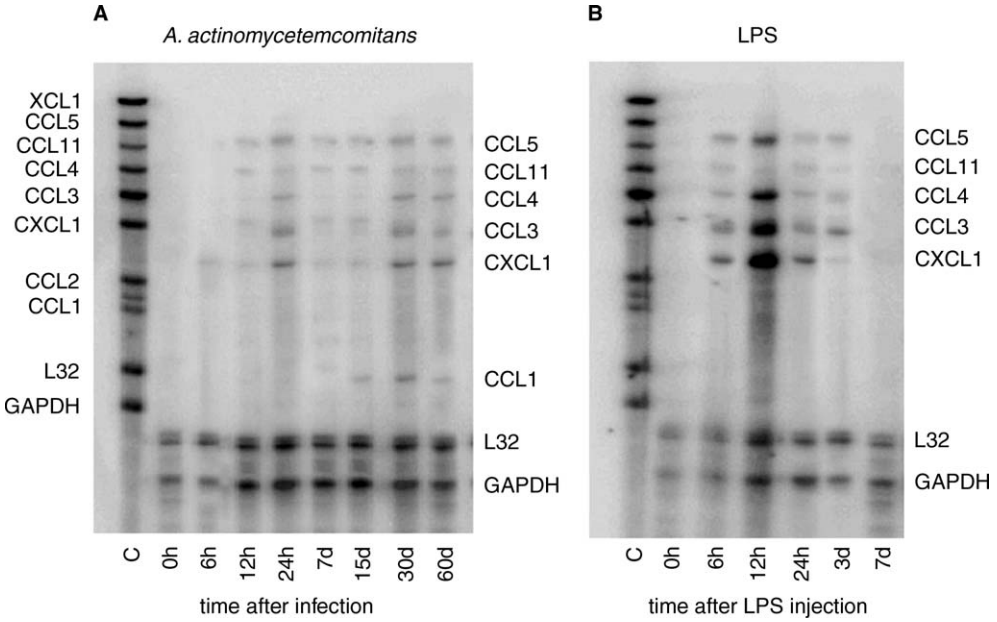


Fig. 2. Kinetics of chemokine mRNA expression in the course of experimental PD. Periodontal tissues of C57BL/6 mice inoculated orally with *A. actinomycetemcomitans* (A) or LPS (B) was analyzed for chemokine expression by RNA protection assay. Total RNA of periodontal tissues was isolated from zero to 60 days after infection or LPS injection and the expression of chemokines was determined as described in Section 2. The results shown are representative of two independent experiments.

CCL5, CCL11, CCL4, CCL3, CXCL1, CCL2 and CCL1 in gingival tissues of mice from all experimental groups. CXCL1 was the first chemokine to be detected, starting 6 h after initial inoculation and increasing its level of expression after 24 h. The expression of CXCL1 was maintained until 60 days pi. The messages for CCL3, CCL4, CCL5, and CCL11 were also detected 12 h after infection. Expression of these mediators was found to be increased 24 h pi, and was present throughout the period of observation. The kinetics of expression of mRNA for CCL1 were different from those of all other chemokines analyzed, being detected only 15 days after infection. No messages for XCL1 and CCL2 chemokines were detected throughout the period analyzed (Fig. 2A). The injection of LPS in the palatal gingival tissue induced messages for CCL5, CCL11, CCL4, CCL3 and CXCL1 after 6 h, with the expression peaking at 24 h. Infected mice only weakly expressed these chemokines from 24 h up to 3 days (Fig. 2B) pi. Injection of LPS did not induce expression of the chemokines XCL1, CCL2 and CCL1. No messages for any of the chemokines analyzed in this present work were detected in sham-infected and control groups (data not shown). The expression of the housekeeping genes L32 and GAPDH is also shown (Fig. 2).

### *3.3. Kinetics of chemokine and chemokine receptor mRNA expression*

We next evaluated the expression of the chemokines CXCL3 and CXCL10, and chemokine receptors in periodontal tissues by means of RT-PCR. CXCL3 was strongly expressed from 6 to 48 h pi. Message for CXCL10 was detected from 24 h up to 30 days after infection (Fig. 3A). After injection of LPS expression of CXCL3 and CXCL10 was also observed at 6, 24 h, 3 and 7 days after LPS injection (Fig. 3B). RT-PCR reactions for XCL1, CCL5, CCL11, CCL4, CCL3, CXCL1 and CCL1 confirmed the data obtained by RPA (data not shown). No messages for the analyzed chemokines were detected in sham-infected and control groups (data not shown). The expression of the chemokine receptors CXCR3, CCR5, CCR4 and CCR8 was also evaluated after infection with *A. actinomycetemcomitans*. Messenger RNA for CXCR3 and CCR5 was detected after 48 h pi, persisting through the entire period of observation. Infected mice expressed CCR4 and CCR8 mRNA at a later

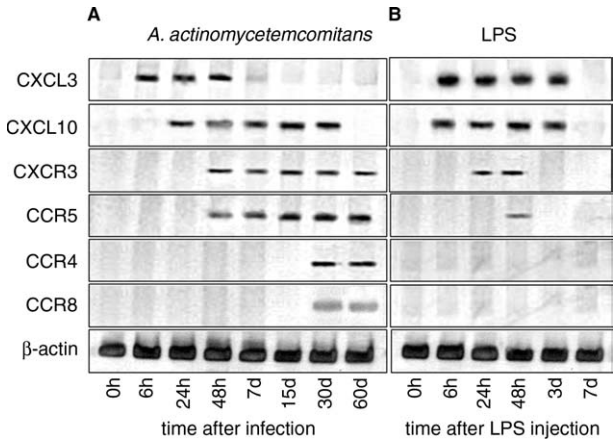


Fig. 3. Kinetics of CXCL3, CXCL10, and chemokine receptor mRNA expression in the course of experimental PD. Periodontal tissues of C57BL/6 mice inoculated orally with *A. actinomycetemcomitans* (A) or LPS (B) were harvested from zero (before infection) until 60 days of infection. The RNA was isolated and the transcripts for CXCL3, CXCL10, CXCR3, CCR5, CCR4, and CCR8 were detected by RT-PCR as described in Section 2. The results shown are representative of three independent experiments.

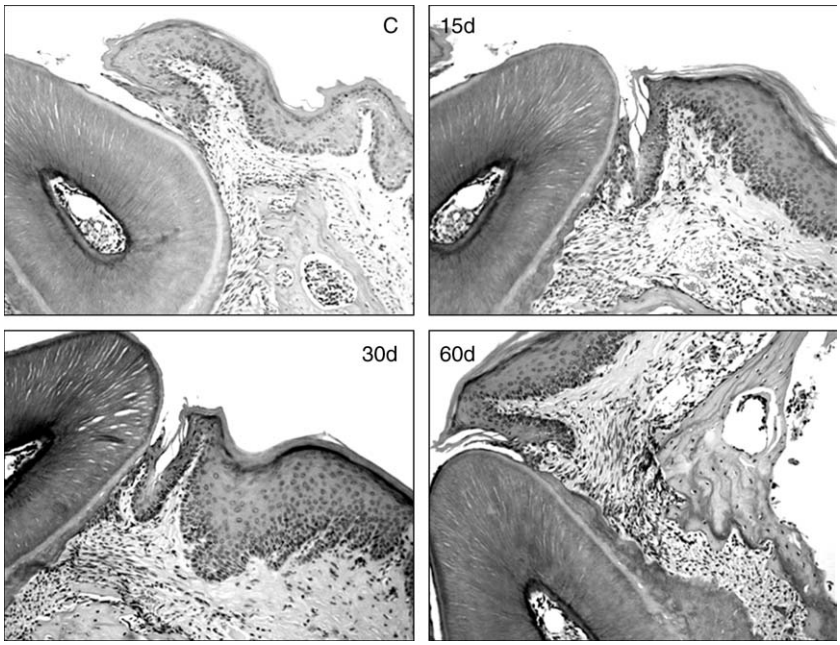


Fig. 4. Histological sections of periodontal tissues of *A. actinomycetemcomitans* infected mice. Periodontal tissue samples of C57BL/6 mice inoculated orally with *A. actinomycetemcomitans* were taken before (C) and at 15 (15 days), 30 (30 days) and 60 (60 days) days after infection. The periodontal tissues were fixed in paraformaldehyde, demineralized in EDTA solution, and embedded in paraffin. Serial sections (5 μm) were cut and histological assessment was carried out following routine hematoxylin and eosin staining. Magnification, ×200.

time point, 30 and 60 days after the first inoculation (Fig. 3A). The expression of CXCR3 and CCR5 in the gingival tissue of mice that received LPS was observed, respectively, at 24 and 48 h, while the expression of CCR4 and CCR8 was not detected at any time point. Messages for the chemokine receptors analyzed were not detected in sham-infected and control groups (data not shown). The expression of the housekeeping gene β-actin is also shown (Fig. 3).

### 3.4. Inflammatory infiltrate in periodontal tissues

A significant inflammatory cell infiltrate was found in the connective tissue proximal to the junctional epithelium and surrounding bone tissue of mice inoculated with *A. actinomycetemcomitans*, extending to all the gingival tissue surrounding the molars (Fig. 4). Inflammatory cells were found to have infiltrated periodontal tissues at 15 days post initial inoculation and such cell migration became more evident at 30 and 60 days post infection. Proliferation of the cells that comprise the junctional epithelium was also seen, appearing on day 15, and persisting up to 60 days post infection. In fact, the infected mice presented a significant increase in the number of leukocytes extracted from periodontal tissues at 15 days ($P < 0.05$), 30 days ($P < 0.05$) and 60 days ($P < 0.01$) post initial inoculation, when compared with sham-infected and non-infected mice (Fig. 5A). By FACS, we determined that PMNs were the predominant cell type in periodontal tissues 24 h pi, reaching a peak at 7 days post infection. These cells were still found in substantial numbers until day 60 pi (Fig. 5B). After day 15 pi, there was a shift towards a predominance of mononuclear cells, comprising $CD4^+$ (Fig. 5C) and $CD8^+$ T cells (Fig. 5D), and $F4/80^+$ cells (Fig. 5E). This infiltrate remained constant until day 60 days pi. In addition, the number of B cells was significantly increased ($P < 0.05$) at day 30 and 60 pi (Fig. 5F).

### 3.5. Analysis of the alveolar bone resorption

We finally evaluated the alveolar bone level, in order to establish the effectiveness of experimentally-induced PD. While sham-infected and non-infected mice did not present alveolar bone loss in the analyzed period (Fig. 6), mice that received oral inoculation of *A. actinomycetemcomitans* presented an evident horizontal bone loss (Fig. 6A), verified by the increase in the area between the CEJ and the ABC at days 30 ($P < 0.05$) and 60 ($P < 0.01$) pi (Fig. 6B). Besides the horizontal bone loss, we also identified infra-bone lesions in the proximal faces, as well as lesions in the inter radicular area of increasing severity in the first and third molars, 30 and 60 days pi (indicated by arrows in Fig. 6A).

## 4. Discussion

In the present study we showed that mice submitted to oral inoculation with *A. actinomycetemcomitans* developed PD, characterized by expression of mRNA for several cytokines, chemokines, and chemokine receptors in the gingival tissues, migration of lymphocytes, macrophages and PMN cells to periodontal tissues, besides marked bone resorption. Some advantages of this model are that the PD is induced by a known periodontopathogen, the time of infection is precise, and there is no requirement for silk ligatures, allowing the study of the effects of the microorganism without the inter-

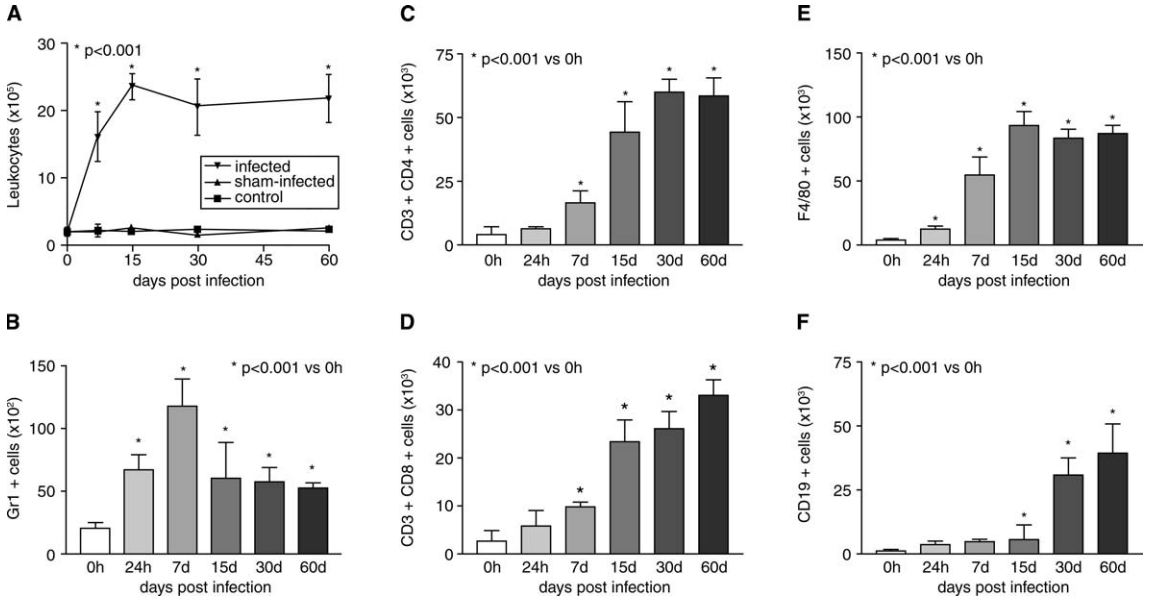


Fig. 5. Phenotypic analysis of the inflammatory cells harvested from *A. actinomycetemcomitans* infected periodontal tissues. C57BL/6 mice were inoculated orally with *A. actinomycetemcomitans*, tissue samples were taken, and the leukocytes isolated from the inflammatory infiltrate as described in Section 2. The cell viability was assessed by Trypan blue exclusion and the (A) total leukocytes counted in a Neubauer chamber. For immunofluorescence staining, PE- and FITC-conjugated Abs specific to GR1, CD4, CD8, F4/80, CD19, and respective control isotypes were employed and analyzed by flow cytometry. The values (mean + S.D.) obtained from three animals at each time point are shown, from two independent experiments. $P < 0.001$, Mann–Whitney test.

ference of mechanical trauma to the tissues. In fact, the oral inoculation results in effective colonization of mouse tissues by *A. actinomycetemcomitans*, which is detected by PCR in periodontal tissues throughout the course of the disease (data not shown) [28].

During the course of the infection we consistently detected in periodontal tissues of mice messages for the pro-inflammatory and Th1-type cytokines TNF-α, IFN-γ and IL-12 [21,22,29,30]. In addition, starting at 30 days post infection, we detected the expression of IL-10 mRNA, an anti-inflammatory cytokine involved in Th2 responses [31]. IL-4, a key cytokine in the induction of Th2 responses [32,33], was not detected, possibly due to the short-half life and instability of its mRNA [34]. In spite of some conflicting data on the role of Th1 and Th2 cytokines in PD, they have been associated, respectively, with activation and suppression of disease activity [30,35–37]. The development of such sequential patterns of cytokine expression was previously reported in response to inoculation of LPS in periodontal tissues of mice [38], although their relevance to cell migration and to the severity of disease outcome was not established.

The first messages for chemokines detected in periodontal tissues of infected mice were for CXCL1 and CXCL3, both involved in the chemoattraction of PMNs [14,39–41]. These chemokines are the murine analogues of human IL-8, which is widely expressed in diseased periodontal tissues [17,18].

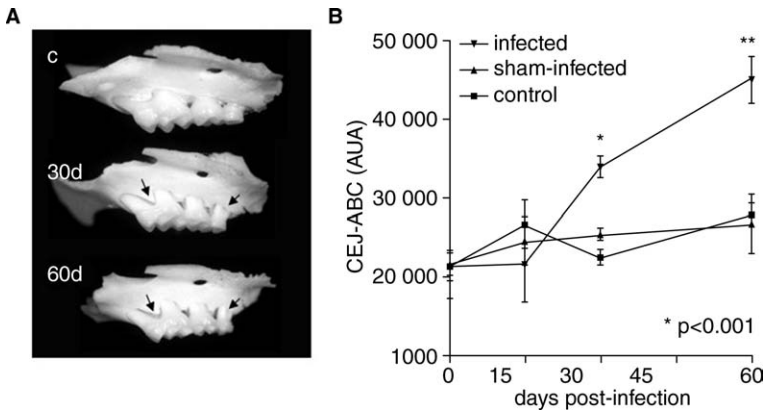


Fig. 6. Alveolar bone loss in *A. actinomycetemcomitans* infected mice. C57BL/6 mice were inoculated orally with *A. actinomycetemcomitans*, periodontal samples were taken and the alveolar bone level was evaluated. A) Maxillar palatal aspect of alveolar bone loss in *A. actinomycetemcomitans* infected mice. The much greater loss of alveolar bone at 30 and 60 days after infection compared with non-infected mice (C) is evident and indicated by arrows. The maxillas depicted were chosen from mice representative of the respective group. B) Alveolar bone loss quantification was performed through the measurements of CEJ–ABC area in the palatal face of maxillary molars, with ImageToll2.0 software. Values (mean + S.D.) obtained from five animals at each time point, from three independent experiments. *$P < 0.05$, **$P < 0.01$, one-way ANOVA, Bonferroni post test.

The expression of CXCL3 was detected only until 48 h post infection while CXCL1 was found at all the time points studied up until 60 days. Accordingly, we found GR1-positive cells at all the time points investigated, as occurs in the acute inflammatory reaction and even in the chronic stages of PD [11,12]. PMNs are thought to play important roles in the pathogenesis of PD, since either hypo or hyper activity of this cell type is associated with severe forms of PD [8,42,43].

The expression of CCL3, CCL4 and CCL5, its receptor CCR5, as well as CXCL10 and its receptor, CXCR3, was also found during all the course of disease. Expression of these chemokines and receptors correlated with the presence of macrophages and CD4 and CD8 lymphocytes. Indeed, macrophages express CCR5, whereas CD4 and CD8 T lymphocytes express CCR5 and/or CXCR3 [14,44,45]. Such cells, chemokines and chemokine receptors are commonly found in diseased periodontal tissues [11,18,20,46], and are known to be involved in Th1-type diseases, such as multiple sclerosis and rheumatoid arthritis [44,47–49]. In fact, rheumatoid arthritis shares several features with PD, including the chronic nature of the inflammatory reaction and bone resorption activity [24]. We also detected in diseased periodontal tissue the expression of the chemokines and chemokine receptors closely associated with Th2 responses. CXCL11 and CCL1, which are detected, respectively, after 24 h and 15 days post inoculation are involved in the chemoattraction of eosinophils/basophils and of Th2 lymphocytes [14,50]. In accordance, the messages for CCR4 and CCR8, both expressed by Th2 cells [14,44,50–52], are expressed only after day 30 pi. These results suggest that the production of different cytokines and chemokines in the diseased periodontal tissues leads to a migration of distinct T helper subsets in the course of experimental PD. Moreover, the pattern of immune response found in mice infected with *A. actinomycetemcomitans* evolves from Th1 to a mixed Th1/Th2 pattern.

The initial occurrence of a Th1 response may be induced by *A. actinomycetemcomitans*, a potent inducer of IL-12 and IFN-γ in vitro [53,54]. This initial Th1 response could be sustained and amplified by the expression of CCL3, CCL4, CCL5 and CXCL10, which attracts $CCR5^+$ and $CXCR3^+$ cells, including Th1 lymphocytes and macrophages [14,47]. Macrophages, found in large numbers in inflamed gingival tissues, are thought to play a significant role in the killing of pathogens and in the release of pro-inflammatory mediators, such as TNF-α, IL-1 and nitric oxide [11,29,55,56]. These mediators also enhance the cellular immune response, which may be useful in controlling invasive pathogens, such as *A. actinomycetemcomitans*. On the other hand, the inflammatory products widely produced by macrophages are known to induce bone resorption by promoting the differentiation and maturation of osteoclasts [29,56]. In this way, the chemoattraction of macrophages may contribute towards the maintenance of the chronic inflammatory reaction and the tissue destruction seen in PD. Additionally, the chemoattraction of IFN-γ-producing Th1 cells, classically involved in the activation of macrophages [57–59], could also contribute to disease progression. This possibility is compatible with the evidence that the adoptive transfer of Th1 cells results in alveolar bone resorption in mice [35].

Following the course of PD in mice, our results showed the expression of CCL1, a known attractant of cells that express CCR4 or CCR8, which can include Th2-type cells and $CD4^+CD25^+$ regulatory T cells [13,14,44,50–52,60]. The Th2-type cytokines in the lesion may enhance the humoral immune response, which in turn may contribute to the control of periodontopathogens in the gingival crevice and in periodontal tissues. In fact, we found a significant increase in the number of B cells in periodontal tissues 30 days after the infection, a finding supported by studies showing accumulation of B lymphocytes in chronic periodontal lesions in humans [11]. Additionally, Th2 cells, which produce the anti-inflammatory cytokines IL-4, IL-10 and IL-13, are associated with suppression of bone resorption [31,37,61,62]. Indeed, the adoptive transfer of cells with a Th2 phenotype to nude rats attenuates the severity of PD [36]. A further potential source of IL-10, a cytokine detected in periodontal tissues 30 days post infection, could be a $CD4^+CD25^+$ T cell subpopulation with suppressive properties, that characteristically express CCR4 and CCR8 [63,64], both receptors found in the periodontal tissues of *A. actinomycetemcomitans* infected mice. Interestingly, during the predominance of the Th1-type response (until 30 days pi) there was an increase in the numbers of inflammatory cells and intense bone loss. By contrast, after the rise in the expression of Th2-type mediators (after 30 days pi) the number of inflammatory cells remained constant. These findings suggest that the pattern of immune response could determine the stable or progressive nature of the lesion. In agreement, in a previous study we found that a predominant expression of Th2-type chemokines and IL-10 was associated with chronic periodontitis, while the expression of Th1-type chemokines and of IFN-γ predominated in aggressive periodontitis [20].

Taken together, our results suggest that cytokines and chemokines are involved in the immunopathogenesis of PD, by driving the selective migration of distinct cell types and by promoting the maintenance of specific leukocyte subsets in the periodontal tissues. In addition, we demonstrate that C57BL/6 mice submitted to oral inoculation of *A. actinomycetemcomitans* represent a useful experimental model of human PD since they develop the characteristic features of this condition. However, the possible influences of the diet, normal oral flora, and the genetic background of mice on the development of the disease are not known. Since C57BL/6 mice are known to develop a predominant Th1 type response [65], it is possible that this model could be not totally reproduced in other mouse strains.

Finally, knowledge regarding the role of cytokines and chemokines in the outcome of PD may provide the basis for future therapeutic interventions, in order to limit the inflammatory process while improving rapid repair of periodontal tissues.

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