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ORIGINAL ARTICLE

Periodontal Injection of Lipopolysaccharide Promotes Arthritis Development in Mice

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Abstract— This study evaluated the arthritogenic effect of lipopolysaccharide (LPS) in a mouse model of periodontal disease. Periodontitis was induced in wild-type CD1 mice by nine LPS injections (10 or 50 ng) into the maxillary mucosa. Untreated mice or injected with LPS at the tail were used as controls. Two weeks after final inoculation, mice were sacrificed to collect blood, maxilla, and paw samples. Development and progression of periodontitis and arthritis were monitored using clinical assessment, micro-computed tomography (micro-CT), ultrasound (US), and histological analysis. CXCL1, IL-1β, IL-6, TNF-α, and anti-citrullinated peptide antibodies (ACPA) serum levels were determined by enzyme immunoassay. Ankle swelling and inflammation manifested after the 5th periodontal injection of 50 ng of LPS and progressed until the end of experiments. Periodontal injection of 10 ng of LPS and LPS tail injection did not induce paw changes. Therefore, the subsequent assessments were conducted only in mice periodontally injected with 50 ng of LPS. Maxillary micro-CT and histological analysis showed that LPS-induced alveolar bone resorption and vascular proliferation in periodontal tissue, but not inflammation. US and histology revealed increased joint space, leukocyte infiltration, synovial proliferation, and mild cartilage and bone destruction in the paws of mice orally injected. Cytokines and ACPA showed a trend towards an increase in LPS mice. This study shows that arthritis and periodontal disease can co-occur in wild-type mice after periodontal injection of LPS at optimal dose. Our model may be useful to improve the understanding of the mechanisms linking periodontitis and arthritis.

KEY WORDS: periodontitis; arthritis; inflammation; mouse model; periodontal disease; animal model.

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INTRODUCTION

Periodontitis is a very common oral chronic inflammatory condition characterized by alveolar bone resorption, periodontal pocket formation, and damage to the gingival tissue and periodontal ligament, which can lead to tooth loss [1, 2].

Different studies have indicated that inflammatory and immune reactions occur in response to pathogenic bacteria accumulation, initially around the gingival margin, and then into the periodontal pocket that progressively forms between

the gums and the teeth. It was shown that the Gram-negative anaerobe *Porphyromonas gingivalis*, through lipopolysaccharide (LPS) and other virulence factors, is one of the major effectors in the development and progression of periodontitis [3–6]. *In vivo* experiments have demonstrated that LPS periodontal treatment induces cytokine release from inflammatory cells, osteoclastic-cell differentiation, and mainly alveolar bone resorption [7–10].

In recent years, it has been proposed that periodontal pathogens, as well as inflammatory products from periodontium, may have access to the blood stream causing systemic effects and/or contributing to systemic diseases, such as diabetes mellitus, adverse pregnancy outcomes, risk of cardiovascular disease, and rheumatoid arthritis (RA) [11, 12]. In this context, epidemiological and clinical evidence has shown higher prevalence and severity of RA in patients with periodontitis and *vice versa*, in comparison to controls [13–16]. This is supported by other studies that have highlighted many common features and risk factors between the two diseases. In fact, both display similar destruction of fibrous connective tissue and bone, and maintenance of inflammation characterized by leukocyte infiltration and production of pro-inflammatory cytokines, notably tumor necrosis factor (TNF)-α [17, 18]. In addition, it has been supposed that RA and periodontitis may share susceptibility genes and environmental factors, such as smoking and obesity [19–24]. Finally, growing evidence indicates that increased levels of anti-citrullinated peptide antibodies (ACPA) and abnormal production of pro-inflammatory cytokines observed in both conditions could represent a mechanistic link between periodontitis and arthritis [17, 18, 25].

Recently, different studies have been conducted using *in vivo* models to investigate the ways in which both diseases interact and influence each other. It has been demonstrated that pre-existing *Porphyromonas gingivalis* oral infection exacerbated collagen-induced arthritis (CIA) in mice, as manifested by accelerated development and enhanced severity of the disease. Interestingly, this phenomenon was associated with increased systemic and local levels of citrullinated peptides and pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, TNF-α, and IL-17 [26–30]. Similar results were obtained when periodontitis was induced both simultaneously and also after collagen immunization, or collagen antibody injection, in mice and rat models [30–33]. Moreover, using SKG mice, which spontaneously develop chronic arthritis, severe swelling of the ankle joint and increased serum levels of ACPA, metalloproteinase (MMP)-3, IL-2, IL-6, chemokine (C-X-C motif) ligand 1 (CXCL1), and macrophage inflammatory protein (MIP)-α were observed after intraperitoneal injection of *Porphyromonas gingivalis* [34]. More recently, severe joint damage accompanied by enhanced pro-inflammatory cytokine production was noticed after bacterial infection in mice with antigen-induced arthritis [35]. Although these *in vivo* observations have demonstrated that periodontitis aggravates experimental arthritis, there are no animal models that mimic the co-occurrence of these diseases without immunization or genetic conditioning.

This study aimed to investigate whether injection of the bacterial component LPS may display arthritogenic effect in a mouse model of periodontal disease.

MATERIALS AND METHODS

Animals

Male, wild-type, CD1 mice were maintained on standard laboratory diet and water *ad libitum* at the Experimental Surgery Center of Padova University. All animals used for the experiments were aged 8–9 weeks and weighed 30–35 g. All experimental procedures were approved by the Padova University Animal Ethic Committee and Italian Ministry of Health.

Experimental Periodontitis

Periodontitis was induced in CD1 mice by injection of 10 or 50 ng of LPS (Sigma-Aldrich, Milan, Italy) in 5 μl of PBS every 48 h into the vestibular gingiva of the second molar on the left maxilla. Alternatively, a group of mice were injected with PBS only using the same procedure. In addition, untreated mice or injected every other day with 50 ng of LPS into the tail were used as controls. Each group consisted of 10 mice that were monitored daily for clinical signs. Assessment of arthritic changes in limbs was estimated by conventional visual scoring method (scale 0–5) [36] and recording the paw swelling with a digital caliper (Kroeplin, Schlüchtern, Germany). Two weeks after the 9th injection, mice were sacrificed by CO_2 exposure and maxilla and paw samples were removed and fixed in 4% (*w*/*v*) paraformaldehyde. In addition, blood was collected by cardiac puncture and centrifuged at 1500 rpm for 10 min at 20 °C (ALC PK 130 centrifuge, rotor No. T535) to obtain serum.

Micro-computed Tomography Analysis

Each maxilla (left and right) was placed in a cylindrical polyethylene container (*i.e.*, diameter of 1.1 cm) and

then analyzed by an *ex vivo* high-resolution micro-computed tomography (micro-CT) 1172 (Skyscan, Aartselaar, Belgium). The following micro-CT parameters were applied: 89 Kv, 112 μA, 1 mm aluminum filter, 9 μm isotropic voxel size, and 1280 × 1024 field of view. The acquired raw data were reconstructed with the N-Recon Software (Skyscan, Aartselaar, Belgium). The bitmap images obtained after the reconstruction were then converted in Dicom (Dicom Converter, Skyscan, Aartselaar, Belgium). Volumetric 3D and multiplanar reconstructions (MPR) were performed with Horos (Open Source Software, https://www.horosproject.org). The distance between the cementum-enamel junction (CEJ) and the alveolar bone crest (ABC) (Fig. 1a) of each molar (*i.e.*, on the mesial and distal side) was measured on the sagittal plane using the MPR images.

Evaluation of Arthritic Joints by Ultrasound

Ultrasound (US) evaluation was performed, after the sacrifice, on the knees and ankles of all mice using a 15-MHz probe (LOGIQ S8, General Electric Company) and joint space was measured (Fig. 1b). Each knee and ankle was placed in a plastic tub filled with water. The transducer was placed in the water and was kept by a bracket in a firm position at 10 mm above the selected joint.

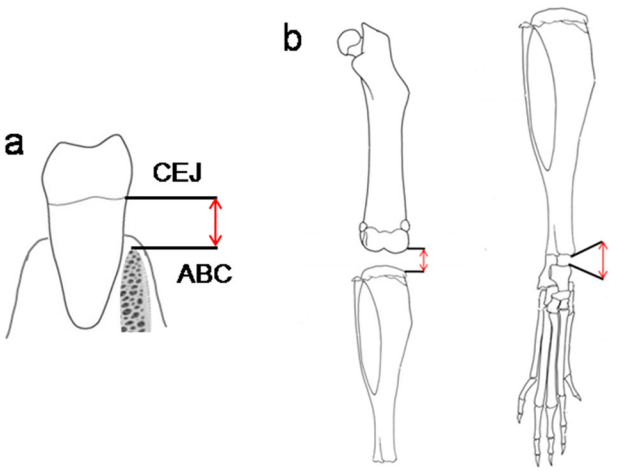

Fig. 1. Representative illustration of measurements taken. **a** The distance between the cementum-enamel junction (CEJ) and the alveolar bone crest (ABC) of the molars (*i.e.*, on the mesial and distal side) was measured on the sagittal plane using the multiplanar reconstructions (MPR) images after micro-CT analysis. **b** The tibio-astragalar and tibio-femoral distances of the hind paws were measured by ultrasound.

Histological Analysis

Maxilla and paw specimens were processed for histological analysis. Following fixation, maxilla and hind paw specimens were decalcified in a solution of formic acid, nitric acid, and distilled water for 24 h. Subsequently, they were embedded in paraffin and sectioned at a thickness of 4 μm. Sections were stained with hematoxylin and eosin for histological analysis. Slides were viewed using a Leica DM4000B microscope equipped with a Leica DFC420 camera.

A four-tier system (0–3) was used to evaluate inflammation, vascular proliferation, and bone resorption in the maxillae (0 = normal, 1 = mild effect, 2 = moderate effect, 3 = severe effect). In addition, a quantitative assessment of bone resorption was performed by measuring alveolar bone length on histological sections.

Paws were assessed for cartilage destruction, pannus formation, bone resorption, and inflammatory changes based on the number and type of inflammatory cells in the knee and ankle joints. The severity of each parameter was scored separately on a scale from 0 to 5 (0 = normal, 1 = minimal effect, 2 = mild effect, 3 = moderate effect, 4 = marked effect, 5 = severe effect).

Serum Analysis

ACPA and cytokine levels were measured in serum by commercially available enzyme immunoassay: IL-1β (eBioscience, San Diego, CA, USA), IL-6, TNF-α (BioLegend, San Diego, CA, USA), CXCL1 (Peprotech, Rocky Hill, NJ, USA), and ACPA (Wuhan Fine Biotech Co., Wuhan, China), respectively.

Statistical Analysis

Data are reported as mean ± standard deviation (SD). The Student *t* test was used to determine the statistically significant differences between the experimental groups. A p value < 0.05 was considered significant.

RESULTS

Clinical Assessment

During LPS periodontal treatment, we observed swelling of the left cheek and redness of the left maxillary gingival and palatal regions. The gums were bleeding upon provocation. Swelling and inflammation were noticed in the hind paws after the 5th periodontal injection of 50 ng of LPS, picked at day 18, and continued for the next 14 days with paw thickness and arthritis score significantly higher than

those of untreated mice. The greater swelling was observed especially at the ankles (Fig. 2a–c). Some mice presented deformity and limited mobility in some joints. PBS alone or LPS administered at a dose of 10 ng through the oral mucosa did not induce paw changes. Animals that received LPS tail injections did not show any clinical signs of arthritis. Therefore, the subsequent assessments were conducted only on mice periodontally injected with 50 ng of LPS.

At the sacrifice, the ankle thickness of mice injected into the oral mucosa with 50 ng of LPS was around 1.5-fold higher than that observed in controls (Fig. 2d). Of note, considering the mean of the difference between the final and the initial ankle thickness, the changes were even more evident, displaying a 12-fold increase in mice receiving LPS, as compared with untreated group (Fig. 2e). Concomitantly, arthritis score remained enhanced until the end of the study (Fig. 2f).

Together, these observations show that arthritis symptoms can occur after LPS oral injection in mice.

Assessment of LPS-Induced Periodontitis

The micro-CT analysis revealed more alveolar bone resorption and a greater alveolar crest loss in the LPS-

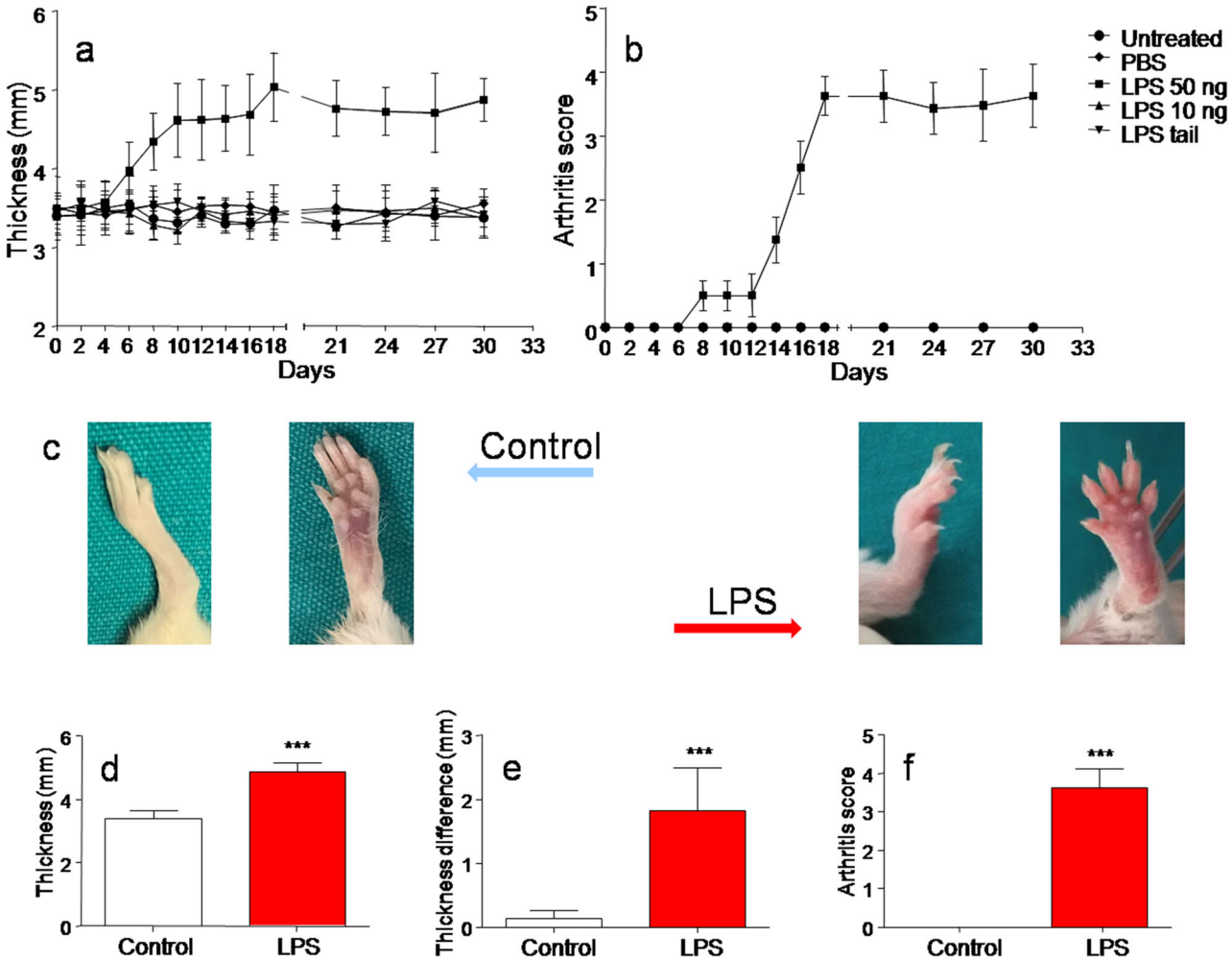

Fig. 2. Clinical evaluation of arthritis. Mice were injected with LPS or PBS only into the gingiva of the left maxilla or into the tail every 48 h for 18 days, and then sacrificed 2 weeks after the 9th injection. Untreated mice were used as controls. Ankle swelling (**a**) and arthritis score (**b**) were evaluated at different time points through the experimental period. **c** Representative images of the hind paws from untreated group and mice periodontally injected with 50 ng of LPS. **d** Ankle thickness, **e** difference between final and initial ankle thickness, and **f** arthritis score were evaluated also at the end of the experiment. Results are presented as mean ± SD of 10 mice per group. ***$p < 0.001$ *vs* control group.

treated maxilla than in the controls (Fig. 3a–d). Indeed, taking into account all the three left molars, the CEJ-ABC distance was greater in the inoculated (0.28 ± 0.09 mm) than in the untreated (0.17 ± 0.06 mm) mice ($p < 0.001$) (Fig. 3e). Similar results were observed in the contralateral non-inoculated maxilla (right side, $p < 0.001$) (data not shown here). The single molar analysis (*i.e.*, mean value of the mesial and distal CEJ-ABC distance of each one of the three left molars separately) confirmed these results since the LPS showed a greater CEJ-ABC distance than the controls in each left maxillary molar ($p < 0.001$, each). Furthermore, the greatest difference occurred in the second molars, as demonstrated by the ratios of the measurements of the LPS and controls (*i.e.*, 1.51, 1.77, and 1.58, respectively, for the first, second, and third molar) (Fig. 3f).

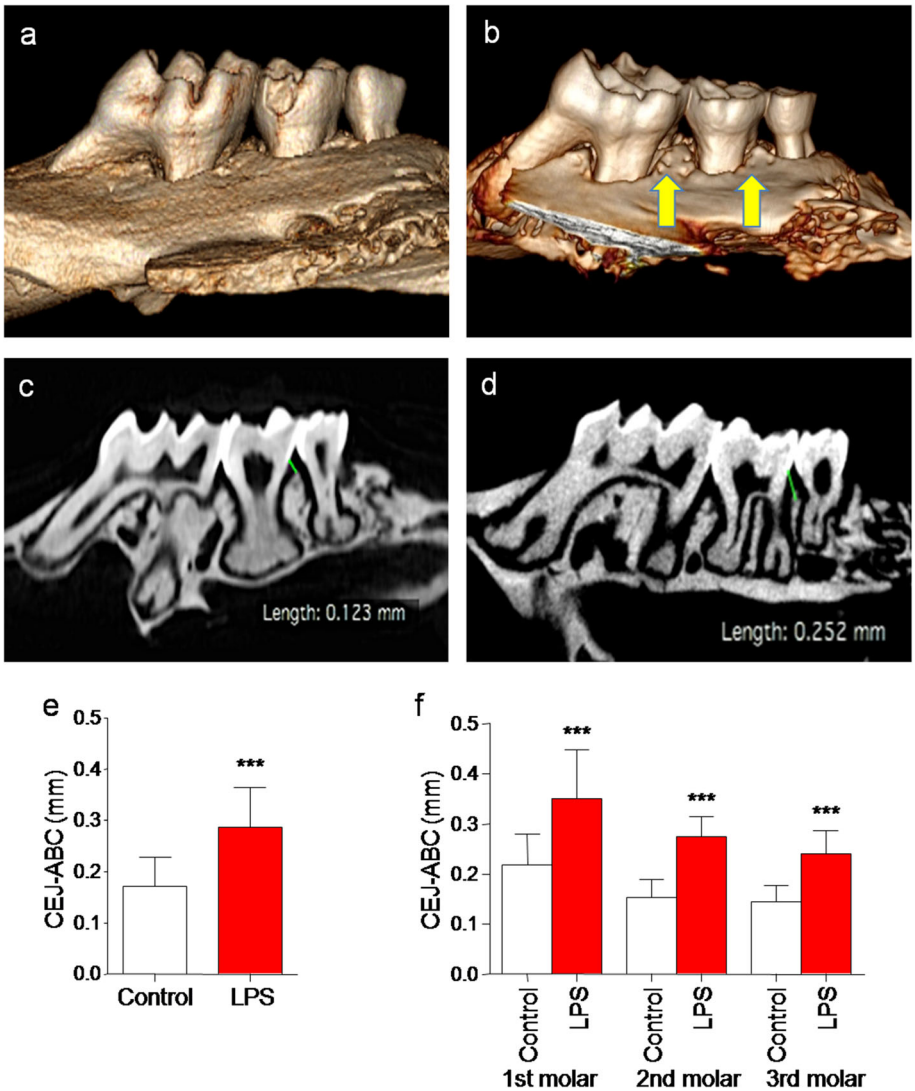

Fig. 3. Periodontal injection of 50 ng of LPS induced reduction of alveolar crest in maxilla of CD1 mice. Micro-CT analysis of the mouse maxilla was carried out at the end of the experiment. Representative 3D volume reconstruction images of the left maxilla of controls (**a**) and of LPS-treated mice (**b**). The maxilla of the LPS-treated mice shows irregularities and resorption of the alveolar crest bone (yellow arrows in **b**). Multiplanar reconstructions (MPR) on the sagittal plane of the left maxilla of one control (**c**) and of one LPS-treated mouse (**d**) showing a greater distance between the cementum-enamel junction (CEJ) and the alveolar bone crest (ABC) on the distal side of the second maxillary molar of the LPS treated. CEJ-ABC distance of all three left molars (**e**) and of each one of the three left molars separately (**f**). Results are presented as mean ± SD of 10 mice per group. ***$p < 0.001$ *vs* control group.

Histological analysis confirmed that LPS induced a reduction of the alveolar crest height accompanied by periodontal pocket formation and a substantial alveolar bone resorption (score 1.8 ± 0.42) characterized by an increase in the irregularity of bone surface and the presence of osteoclasts and lacunae. Interestingly, LPS group showed evidence of a moderate vascular proliferation (score 2.2 ± 0.42) in periodontal tissue, but not inflammation (Fig. 4). In addition, the alveolar bone length resulted significantly decreased in inoculated mice compared to healthy controls (377.139 ± 33.366 *vs* 654.345 ± 33.366 μm, $p < 0.01$).

These results indicate successful induction of periodontitis.

Effects of LPS Periodontal Injection on the Arthritis Onset

At the time of euthanasia, US examination showed the presence of effusion and a 1.5-fold higher joint space in the ankle of mice with periodontitis compared to controls. In addition, some LPS-treated animals presented minimal effusion in the knee joint but not enlargement of the distance between femoral and tibial bone margins (Fig. 5a–e).

The hematoxylin and eosin-stained sections revealed leukocyte infiltration, mostly neutrophils, and synovial proliferation in the ankle joints of LPS-injected animals. The same sections had mild cartilage thinning and bone resorption. Moderate inflammation and pannus formation were observed also in the knee joints of the inoculated mice. Slight differences in the knee cartilage and bone were noticed between the two groups (Fig. 5f – o).

Together, these data strengthen results of Fig. 2 confirming that arthritis can develop after LPS oral injection.

Effect of LPS Periodontal Injection on ACPA and Cytokine Serum Levels

In order to evaluate whether systemic factors might play a part in the development and progression of periodontitis and arthritis, the levels of inflammatory cytokines and ACPA in serum were assessed. As shown in Fig. 6, in arthritic mice, the secretion of CXCL1, IL-6, and TNF-α was increased by 5-, 2-, and 8-fold, respectively, compared to healthy group. Although there was no statistical difference, IL-1β and ACPA levels showed a trend towards an increase in the case of arthritic animals with accompanying periodontal disease.

These results suggest that systemic inflammation can develop after oral injection of 50 ng of LPS by the production of pro-inflammatory factors.

DISCUSSION

In this study, we demonstrate for the first time that arthritis can develop spontaneously and co-occur with periodontitis in an *in vivo* experimental model of periodontal disease. The results obtained show that wild-type mice orally injected with an optimal dose of LPS developed loss of alveolar bone in maxilla and paw swelling, inflammation, and joint damage. These processes were accompanied by an increase of pro-inflammatory factors suggesting that they might play part in the development of disease.

Various animal models of experimental periodontitis have been developed in recent years, using a number of different methods, such as ligatures or sutures infected with bacteria placed around selected molars, injection or topical application into the gingiva, or oral administration of pathogens [37, 38]. In this study, we used LPS injection into the vestibular gingiva of mice. This is an established, direct, and controlled periodontal disease model that has been demonstrated to promote bone resorption, differentiation, and formation of mature osteoclasts, significant inflammation, and vascular response in gingival and periodontal tissue [7–10, 39]. As expected, in our experiments, we observed increased CEJ-ABC distance, alveolar bone resorption, and vascular proliferation in left maxilla, particularly in the second molar region, which coincides with the LPS injection site. Despite gingival swelling, redness, and bleeding were noticed during LPS periodontal treatment, at the time of euthanasia, no appreciable histological signs of inflammation, such as infiltration of inflammatory cells, were observed in periodontal tissue. This might be because we used very low doses of LPS and mice were sacrificed 2 weeks after the last injection. Indeed, other studies indicate great variations in the LPS dosage (20- to 30,000-fold higher than we used) [9, 40–42], and suggest that limitations associated with this model are mainly related to the necessity for constant injections throughout the experimental period [37]. Noticeably, bone resorption and enhanced CEJ-ABC distance were observed also in non-injected maxilla of the inoculated mice. These results demonstrate that the dose of LPS used as well as the injection time are sufficient to induce a generalized periodontal reaction. Intriguingly, in addition to periodontitis, our mice developed spontaneous increase in paw thickness and clinical scores which are phenotypic manifestations commonly

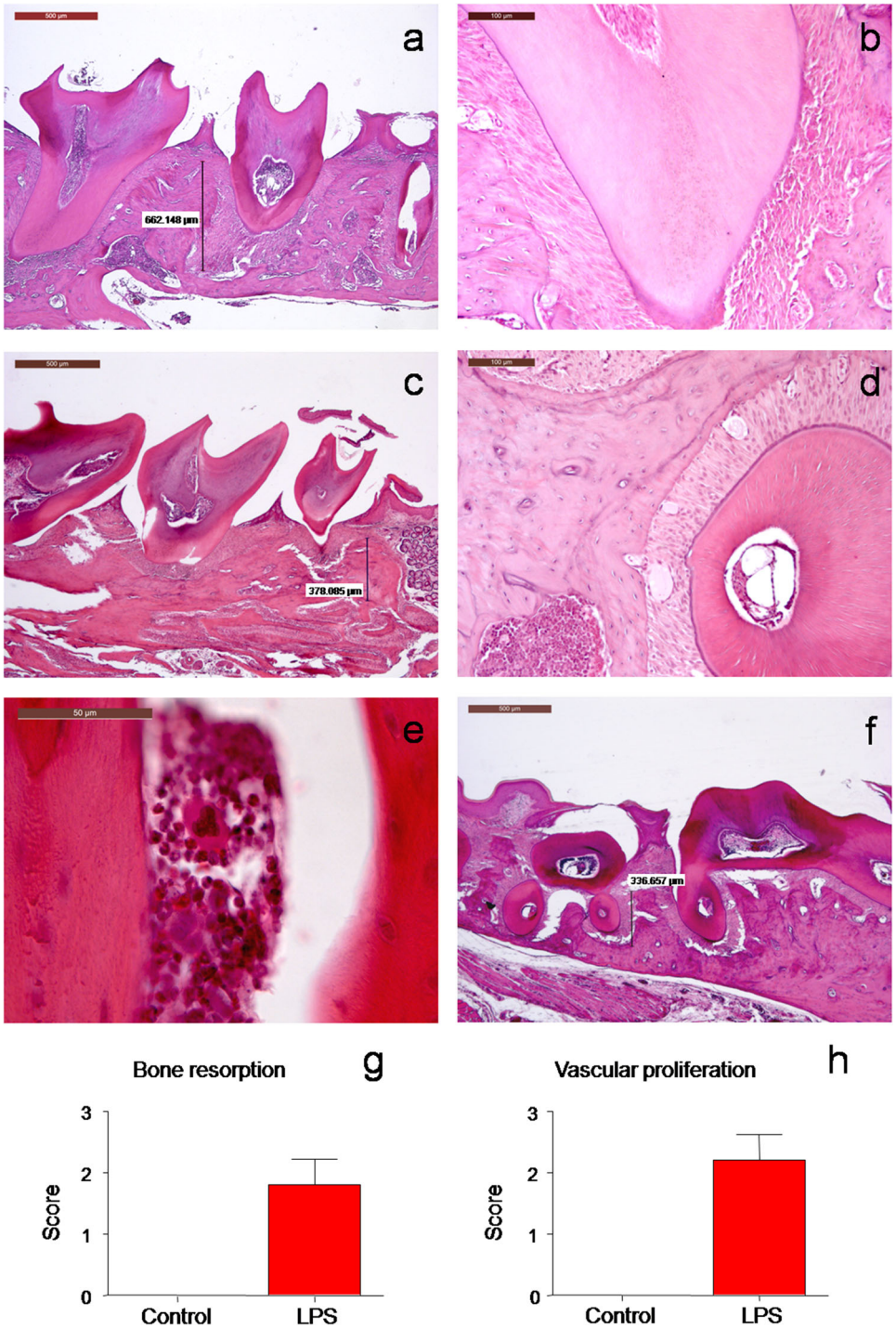

Fig. 4. Periodontal injection of 50 ng of LPS induced histological changes in maxilla of CD1 mice. Histological analysis of the maxillary periodontal tissues was carried out at the end of the experiment. Representative images of the left maxilla of controls (**a**, **b**) and of LPS-treated mice showing a lower alveolar bone crest height and periodontal pocket formation on the maxillary tissue of the inoculated mice (**c**). Evidence of alveolar bone resorption, vascular proliferation (**d**), and presence of osteoclasts (**e**) were observed in LPS group. Histological image of the alveolar bone length in contralateral non-injected maxilla (right) of LPS-treated mice (**f**). Original magnification of **a**, **c**, **f** × 50; **b**, **d** × 200; and **e** × 600. Histological scores for bone resorption (**g**) and vascular proliferation (**h**). Results are presented as mean ± SD of 10 mice per group.

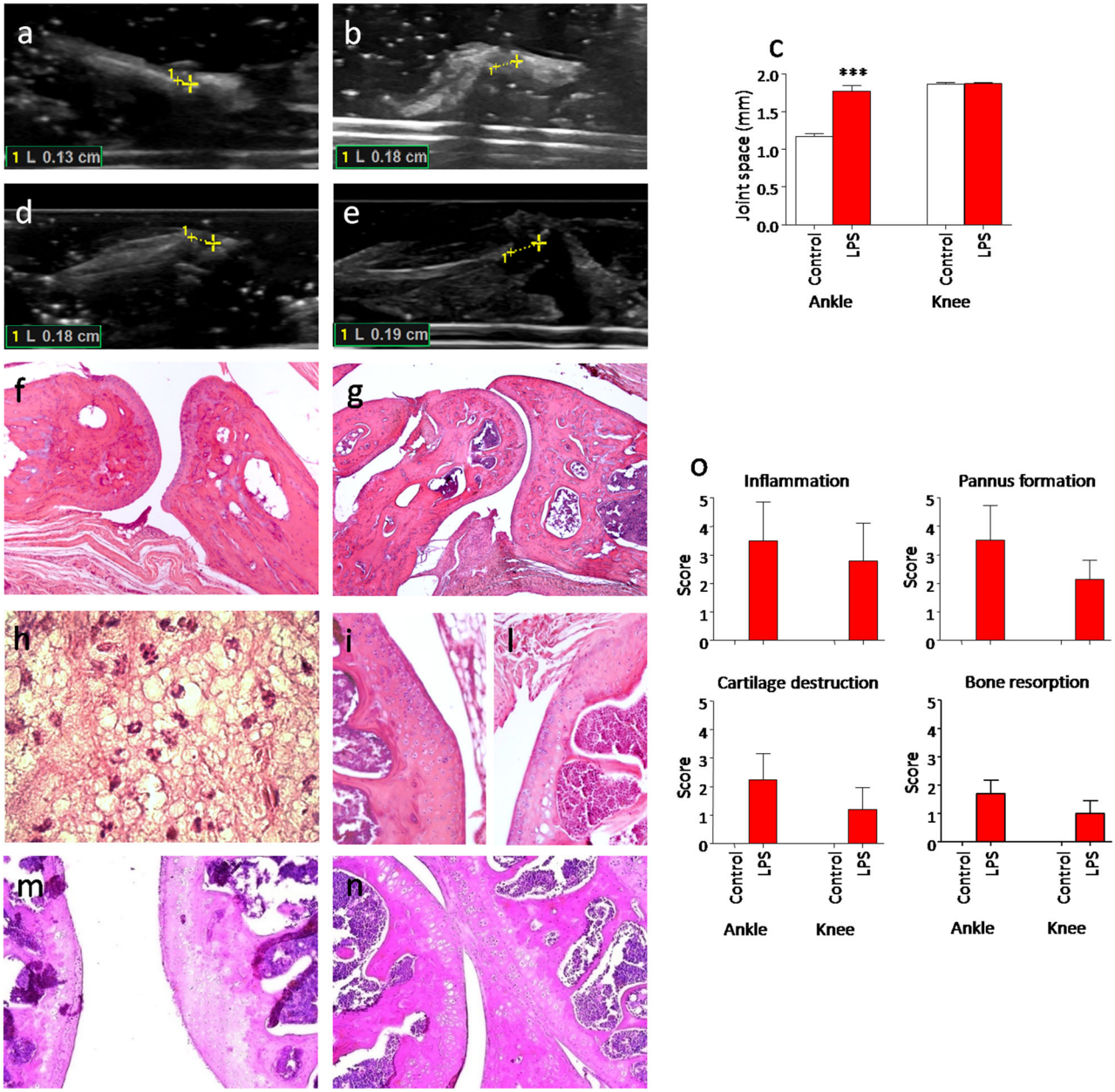

Fig. 5. Arthritis co-occurs with periodontitis after periodontal injection of 50 ng of LPS in CD1 mice. Ultrasound evaluation and histological analysis of the knee and ankle joints were carried out at the end of the experiment. Representative US images of the ankle joint of controls (**a**) and LPS periodontally injected mice (**b**). **c** Ankle and knee joint space. Results are presented as mean ± SD of 10 mice per group. ***$p < 0.001$ *vs* control group. Representative US images of the knee joint of controls (**d**) and LPS periodontally injected mice (**e**). Representative hematoxylin and eosin (H&E)-stained sections of the ankle joint of controls (**f**) and LPS periodontally injected mice (**g**), showing inflammatory cell infiltration, mainly neutrophils in articular tissues of the inoculated mice (**h**). H&E staining of normal and thinned cartilage sections from ankles of controls (**i**) and LPS periodontally injected mice (**l**). Representative H&E-stained sections of the knee joint of controls (**m**) and LPS periodontally injected mice (**n**). Original magnification of **f**, **g** × 50; **h** × 1000; **i**, **l** × 200; and **m**, **n** × 100. **o** Histological scores for inflammation, pannus formation, cartilage destruction, and bone resorption. Results are presented as mean ± SD of 10 mice per group.

observed in mouse models of arthritis. The presence of the disease was confirmed by results from US and histological evaluations that revealed an increase of the joint space and signs of inflammation characterized by leucocyte

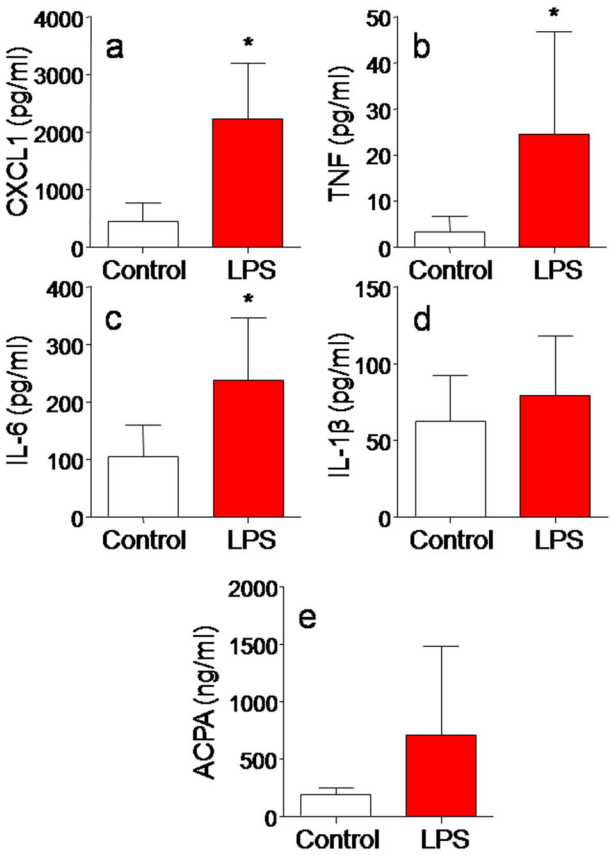

Fig. 6. Effect of periodontal injection of 50 ng of LPS on systemic factors in CD1 mice. Serum from controls and LPS periodontally injected mice were analyzed for CXCL1 (**a**), TNF-α (**b**), IL-6 (**c**), IL-1β (**d**), and ACPA (**e**) by enzyme immunoassay. Results are presented as mean ± SD of 10 mice per group. *$p < 0.05$ *vs* control group

infiltration, accompanied by pannus formation and marginal erosion of the bone and cartilage. To our knowledge, it is the first time that spontaneous arthritis onset has been described in an experimental periodontitis model without any preceding induction. It is not clear whether paw involvement was not manifested in previous reports that used similar animal models, or the authors did not report it. Moreover, only few *in vivo* studies have been conducted so far to investigate the relationship between the two diseases, and they were limited to evaluating the impact of periodontitis on the development of arthritis induced by established conventional methods, such as immunization with collagen or injection of pristane [31, 33, 43].

In our experiments, arthritis was observed after repeated injections into the vestibular gingiva of an optimal dose of 50 ng of LPS, and the ankle was the most severely affected site with an increase of all clinical parameters and histological scores evaluated. In particular, this joint exhibited greater swelling, synovial proliferation, and synovial effusion associated to a higher joint space than those recorded in the knee. Even though some mice presented severe deformity that also led to permanent loss of the fingers, cartilage and bone resulted only mildly injured in most cases. This might be because longer periods may be necessary in order to cause more severe damage in these compartments.

It has been proposed that chronic periodontitis might influence the development of RA through an increase of citrullinated proteins and subsequent excessive production of ACPA [18]. In our study, although the ACPA levels tended to be increased in LPS-injected mice, they did not reach statistical significance. This might be explained by the use of membrane component of bacteria, rather than whole *Porphyromonas gingivalis*, which has been shown to induce production of ACPA *via* peptidylarginine deiminase (PAD) promotion of peptide citrullination [6]. According to our results, an interesting study by Scher *et al.* reported that patients with new-onset RA exhibited a high prevalence of periodontitis at disease onset, despite this was not correlated with ACPA titers [15]. Therefore, this suggests the involvement of citrullination-independent mechanisms, such as Th17 cell induction *via* IL-1 and IL-6 [28]. In addition, the association between periodontitis and arthropathies has been widely reported in patients with RA, in which ACPA serve as informative biomarkers [44, 45], whereas only few conflicting studies have investigated the possible link between periodontal disease and other forms of arthritis that present lower ACPA positivity, such as psoriatic arthritis, reactive arthritis, or osteoarthritis [46–49].

Overproduction of pro-inflammatory cytokines, in particular TNF-α and IL-6, has been widely described in RA as well as periodontitis [50]. In addition, it has been demonstrated that the aggravation of experimental arthritis induced by oral bacteria is accompanied by increased local and systemic levels of different cytokines, suggesting a potential key role of these inflammatory mediators in linking the two diseases. Indeed, increased levels of IL-17 and TNF-α were observed in the articular tissues of mice with concomitant antigen-induced arthritis and periodontitis [39], and MIP-1α, IL-2, IL-6, and CXCL1 serum concentrations were higher in SKG (genetically predisposed for RA) mice after injection of laminarin and *Porphyromonas gingivalis* [34]. Even though, in our experiments, IL-1β serum levels were not significantly increased in arthritic mice, we observed a strong elevation in CXCL1, IL-6, and TNF-α that are among the main clinical

parameters considered to evaluate inflammation in *in vivo* models of arthritis [51–53]. This altered cytokine balance may be related to the progression of periodontal inflammation through the bloodstream, leading to the immune cell activation at distant sites of the body where they promote tissue injuries.

Although this study does not elucidate the mechanism behind the associations between the two diseases, the premise that LPS injection into the tail did not induce arthritic signs suggests that periodontium may be considered an important way for the activation of immune response and trigger the onset of arthritis even without genetic conditioning. In a context similar to that of our experiments, it has been proposed that LPS can activate specific matrix metalloproteinases which induce damages to collagen-rich tissues: gingiva, periodontal ligament, and alveolar bone [54]. This could lead to the production of collagen debris, which may be transported with specific inflammatory mediators *via* blood or lymphatic circulation from the periodontium to distant joints and recognized as non-self by a primed immune system. The latter hypothesis suggests that LPS itself and associated immune response may mimic the effect of adjuvant, which helps to boost immune responses in several animal models of inflammatory disease, in particular experimental arthritis.

In conclusion, this study demonstrates that arthritis and periodontal disease can co-occur after LPS oral injection in wild-type mice without preceding induction, providing an experimental link between periodontitis and arthropathies. Our model may be useful to improve the understanding of the mechanisms underlying the link between periodontitis and arthritis, and to identify new targets for therapy.

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COMPLIANCE WITH ETHICAL STANDARDS

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

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