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Pre-existing periodontitis exacerbates experimental arthritis in a mouse model

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Abstract

Aims: Previous studies have shown a higher incidence of alveolar bone loss in patients with rheumatoid arthritis (RA) and that patients with periodontitis are at a greater risk of developing RA. The aim of this study was to develop an animal model to assess the relationship between pre-existing periodontitis and experimental arthritis (EA).

Methods: Periodontitis was first induced in mice by oral gavage with *Porphyromonas gingivalis* followed by EA using the collagen antibody-induced arthritis model. These animals were compared with animals with periodontitis alone, EA alone and no disease (controls). Visual changes in paw swelling were assessed to determine clinical development of EA. Alveolar bone and joint changes were assessed using micro-CT, histological analyses and immunohistochemistry. Serum levels of C-reactive protein were used to monitor systemic inflammation.

Results: Mice with pre-existing periodontitis developed more severe arthritis, which developed at a faster rate. Mice with periodontitis only also showed evidence of loss of bone within the radiocarpal joint. There was also evidence of alveolar bone loss in mice with EA alone.

Conclusions: The results of this study indicate that pre-existing periodontitis exacerbated experimental arthritis in a mouse model.

Key words: bone; chronic inflammation; *P. gingivalis*; periodontal disease; RANKL; rheumatoid arthritis

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Periodontitis and rheumatoid arthritis (RA) are two of the most common chronic inflammatory diseases affecting humans (Albandar & Rams 2002, Silman & Pearson 2002). In the past, these two diseases have been studied independently with attempts focused on understanding the individual disease mechanisms and potential therapeutic strategies. However, with close examination, it is clear that both RA and periodontitis have many similarities that warrant further investigation (Bartold et al. 2005, de Pablo et al. 2009).

Both diseases demonstrate an exuberant chronic inflammatory reaction and the presence of large numbers of immune cells including T and B lymphocytes, neutrophils and monocytes. They also have similar cytokine profiles including high numbers of pro-inflammatory cytokines such as TNF-α and interleukins (Bozkurt et al. 2000, Ogrendik et al. 2005). It is this inflammatory reaction that eventually leads to destruction of both the soft and hard tissues of the joint and alveolar bone in RA and periodontitis, respectively. One important common pathway involves the upregulated expression of receptor activator of nuclear factor κB ligand (RANKL) by fibroblasts and lymphocytes, which is an essential factor for osteoclast formation (Crotti et al. 2003, Haynes et al. 2003, Cantley et al. 2009b, Bartold et al. 2010a).

Numerous studies have reported that the relationship between periodontitis and RA may be bidirectional. For example, while many studies have demonstrated significantly higher incidence of tooth loss and alveolar bone loss in patients with RA (Mercado et al. 2000, 2001, Al-Shammari et al., 2005), others have reported that periodontitis is a risk factor for developing RA or even enhancing the severity of RA (Ribeiro et al. 2005, Havemose-Poulsen et al. 2006). More recently, studies have demonstrated that treatment of periodontitis may reduce the severity of arthritis (Ribeiro et al. 2005, Al-Katma et al. 2007, Ortiz et al. 2009). While many possibilities exist to explain these inter-relationships, one process gaining interest is the role that autoim-

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munity to citrullinated proteins might play in the development of RA. Of particular interest is the role that *Porphyromonas gingivalis* might play because this periodontal pathogen can citrullinate proteins through the release of peptidylarginie deiminase (Rosenstein et al. 2004, Wegner et al. 2010).

Our group has recently demonstrated, using a rat model, that the presence of a pre-existing chronic inflammatory reaction induced by a heat killed (non-infective) form of the periodontal pathogen *P. gingivalis*, exacerbated the development of adjuvant arthritis in female DA rats (Bartold et al. 2010b). This established that an extra synovial inflammation, similar to but not identical to periodontitis, exacerbated experimental arthritis (EA). In the present investigation we have extended these studies to include an assessment of an infective component (*P gingivalis*-induced periodontitis) to the development of EA.

Recently a model in which periodontitis and arthritis were both co-induced in an inflammation-prone mouse strain [acute inflammatory reactivity maximum (AIRmax)] and (AIRmin) using the pristane-induced arthritis (PIA) model has been described (Trombone et al. 2010). They found that co-induction in AIRmin mice did not alter the course of both pathologies. Clinical investigations by our group have suggested that patients with severe RA are more likely to have advanced periodontitis and vice versa. Patients with RA taking NSAIDs, which are likely to reduce periodontal inflammation still have signs of periodontal destruction indicating that periodontitis develops and may not be detected before the RA development (Bartold et al. 2005). Hence, to further understand this relationship, an animal model was developed to assess the relationship between pre-existing periodontitis followed by induction of arthritis.

Methods

Animals

Eight-week old female Balb/c mice were obtained from the Institute of Medical and Veterinary Science (IMVS) Animal Services Division and approval was obtained from the Animal Ethics Committees of the University of Adelaide and the IMVS, Adelaide, South Australia. All experiments were carried out according to the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997). For this study a total of 14 mice were used. Group 1 ($n = 4$) consisted of mice induced with periodontitis alone, group 2 ($n = 4$) mice were induced with arthritis only, group 3 ($n = 4$) mice were first induced with periodontitis and subsequently with arthritis, and the control group received no disease ($n = 2$). All animals received kanamycin (1 mg/ml in drinking water) *ad libitum* for a period of 7 days to reduce the native flora and support colonization of the *P. gingivalis* bacteria as has been reported previously (Baker et al. 2000, Bendyk et al. 2009, Kuula et al. 2009).

Periodontitis induction

Three days after antibiotic treatment ceased periodontitis was induced by oral inoculations of *P. gingivalis* bacteria over a period of 44 days (Cantley et al. 2009a). Cultures of *P. gingivalis* bacteria (W50) were grown on anaerobic blood agar plates and stored at 37°C in an atmosphere of $N_2/CO_2/H_2$ (90:5:5) for 3 days, before harvesting. Bacterial cells were suspended in 2 ml of carboxymethyl-cellulose [2% carboxymethyl-cellulose in phosphate-buffered saline (PBS)] as described previously (Bendyk et al. 2009, Cantley et al. 2009a). The viable count of the bacteria was determined to be 24.3×10^{10} colony-forming units/ml and the dry weight was 12.5 mg/ml. The bacteria (0.1 ml in carboxymethyl-cellulose) were swabbed directly on the gingivae of the mice using a small brush. Mice not receiving periodontitis were swabbed with carboxymethyl-cellulose alone. Following each inoculation mice were kept without any food or water for a period of 1 h to ensure effectiveness of the inoculation. Mice were also housed in an environment void of antibacterial products and were fed monitored powdered food throughout the entire experiment. Live *P. gingivalis* bacteria was recovered from gingival tissue as described previously (Bendyk et al. 2009).

Collagen antibody-induced arthritis induction

To induce arthritis, mice were injected intravenously via the tail vein with 150 μl (1.5 mg) of a monoclonal antibody against type II collagen (Chondrex Inc., Arthrogen-CIA® Arthritogenic Monoclonal Antibodies, Redwood WA, USA). For the periodontitis and arthritis group this injection was carried out on day 44 after the induction of periodontitis. Two days later mice were given an intraperitoneal (i.p.) injection of 20 μl (5 μg) of lipopolysaccharide (LPS) (Chondrex Inc.). The dose of LPS used in this study was lower than the dose recommended by the manufacturers. In pilot studies 10 μg of LPS was used to induce disease. The reason for using a lower dose arose from these initial studies, which indicated that the full dose produced a florid arthritic reaction, which prevented the assessment of any co-morbidity factors. By reducing the dose we could still induce a noticeable arthritic response, which then allowed us to fully assess the impact of pre-existing periodontitis on the arthritic response. Control animals (non-arthritis groups) were injected with PBS alone. Following antibody injection mice were monitored daily by two experienced observers for a period of 10 days. This time point was determined from preliminary studies to be the best to assess bone loss and inflammation. Recording of body weight, monitoring of other factors (including dull/ruffled coat, a change in temperament, reduced food/water intake or a reluctance to move) and clinical scoring of the paws for swelling were all conducted daily. To assess the clinical paw swelling each paw was given a score from 0 to 4, to make a total score of 16 for each animal. 0 = normal paw, 1 = mild but definite redness and swelling of the wrist/ankle, 2 = moderate swelling and redness of the wrist/ankle with digit involvement, 3 = severe swelling of the wrist/ankle with multiple digit involvement and 4 = maximum inflammation within the entire paw, wrist/ankle with many digits involved.

Live animal micro-CT scanning

Throughout the experiment, mice were scanned using a live animal micro-CT scanner situated in Adelaide Microscopy (SkyScan 1076, Kontich, Belgium). The specifications used for scanning and machine details have been published previously (Cantley et al. 2009a, b). Mice were scanned at 74 kV/136 mA with a pixel size of 18 μM, 1 mm aluminium filter and frame averaging of 1. These parameters were chosen to minimize the radiation exposure to the animals and significantly reduce scanning time to around 12 min. per animal. Scanning was conducted initially before beginning periodontitis induction to form baseline measurements, again at day 44 after inducing periodontitis but before collagen antibody-induced arthritis (CAIA) induction and finally at the completion of the

study (10 days after inducing CAIA). Before scanning, the mice were anaesthetized via i.p. injection [rat/mouse anaesthetic – 1 ml xylazine, 2 ml ketamine (100 mg/ml), 17 ml of water in the injection, 0.3 ml for a 30 g mouse]. For each scan the mouse was positioned to ensure that both the head and the two front paws were within the scanning area of interest allowing analysis of both alveolar bone and the paws.

CT scans were then re-constructed using SkyScan N Recon program and bone volume (BV) analysis was carried using SkyScan's CTAn program (SkyScan, Kontich, Belgium). For the heads, the three molars and surrounding alveolar bone of the maxilla was chosen as the region of interest. For the paws, 200 slices either side of the radiocarpal joint of the front paws was used as the region of interest for BV analysis. For each area of interest, BV (mm^3), was calculated using CTAn software. The length of the cemento-enamel junction (CEJ) to alveolar bone crest (ABC) in micrometres was also measured at two locations – between the first and second molars and between the second and third molars on three slices for each mouse using CTAn as described previously (Park et al. 2007).

Histological analysis

At the completion of the study (day 54) mice were humanely killed via CO_2 inhalation. All paws and heads were collected, skinned and placed in fixative solution (10% PBS-buffered formalin) for 48 h. Following this, decalcification was carried out for a period of 2 weeks using 5% formic acid and then the specimens were processed for paraffin embedding in preparation for histological analysis. Sections (7 μm) were cut and stained with haematoxylin and eosin (H&E). Histological sections were imaged using the NanoZoomer Digital Pathology (NDP) (Hamamatsu Photonics K.K., Hamamatsu City, Shizuoka Pref., Japan) at $\times$ 40 magnification. Assessments of the radiocarpal joints in the paws and three molars of the maxilla were made. Two histological sections per mouse were scored based on a method of Tak et al. (1997). Two independent observers, blinded to the tissue type, used a 4-point scale. Scoring was based on the numbers of inflammatory cells (lymphocytes, plasma cells neutrophils or macrophages). Normal tissue ($<5\%$ inflammatory cells) was scored a 0, mild inflammation (5–20% inflammatory cells) was scored a 1, moderate inflammation (20–50% inflammatory cells) was scored a 2 and severe inflammation with a massive immune cell infiltration ($>50\%$ of cells) was scored a 3. Bone and cartilage destruction was assessed by: 0 = normal, 1 = mild cartilage destruction, 2 = evidence of both cartilage and bone destruction, 3 = severe cartilage and bone destruction. Pannus formation: 0 = no pannus, 1 = pannus formation.

Gingival tissues of the three molars of the maxilla were also assessed for inflammation and alveolar bone destruction as assessed by measuring the length (μm) of the CEJ to ABC between the first and second molars of the maxilla. This was conducted using the Nanozoomer program (NDP View) length was measured using the linear measurement tool with images at $\times$ 5 magnification.

C-Reactive Protein (CRP) ELISA

Blood samples were collected from mice on the final day of the experiment via cardiac puncture. Samples were left at room temperature to clot for 2 h, followed by centrifugation at 1000 *g* for 20 min. Serum was collected and stored at $-80^{\circ}C$. A commercially available CRP kit (Mouse CRP Elisa test kit, Life Diagnostics, West Chester, PA, USA) was used to assess the CRP levels in the serum samples. Samples were diluted 1 in 100 and manufacturer's instructions were followed.

Immunohistochemistry

Immunohistochemistry was conducted on paraffin embedded sections to determine the expression of RANKL in the radiocarpal joints and periodontal tissues. Sections were stained with a goat polyclonal antibody (2 μg/ml) against RANKL (Santa Cruz sc-7628, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Negative controls consisted of normal goat serum. A ready to use kit (Vectastain, Vector laboratories, Burlingame, CA, USA) was used for staining sections followed by a brief counterstaining with haematoxylin and lithium carbonate and mounting using aquatex. RANKL staining was scored by a semi-quantitative analysis, by two blinded individuals. A 0–4 scoring system was used as previously described (Kraan et al. 1999). Three areas were scored for each stained section of both periodontal tissues and the radiocarpal joints. The score 0 = 0–5% positive stained cells, 1 = 6–10%, 2 = 11–25% 3 = 26–50% and 4 > 50% positive staining cells.

Statistics

Unpaired *t*-tests were used to analyse statistical differences between the groups. Statistical significance was accepted when $p<0.05$.

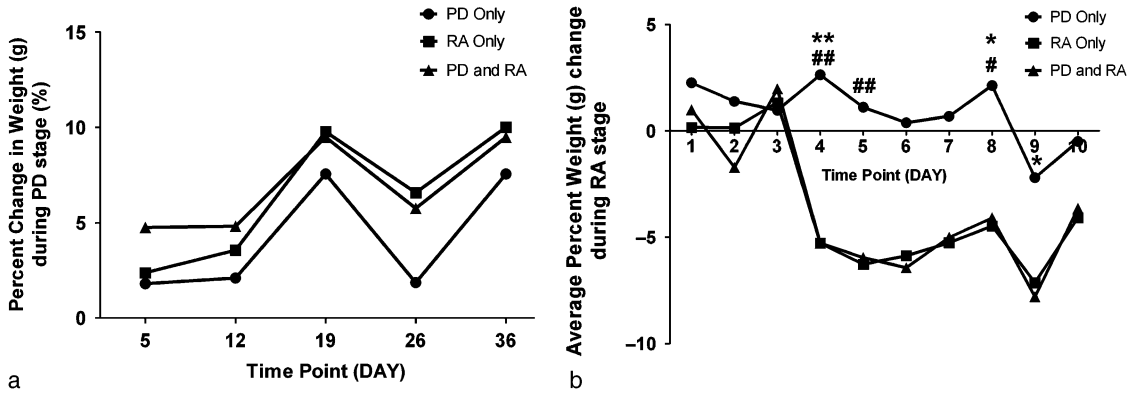

Fig. 1. Average percentage change in weight (grams). (a) The periodontitis induction stage from day 0 to day 36. Weight change relative to day 0. No significant differences between groups at any time points and (b) the collagen antibody-induced arthritis [experimental arthritis EA] stage, (days 0–10) weight change relative to day 0. Data represented as mean ± SEM. ($n = 4$ per group) $^{**}p<0.01$, $^{*}p<0.05$ periodontitis and EA compared with periodontitis alone, $\#\#p<0.01$ $\#p<0.05$, EA alone *versus* periodontitis alone.

Results

Weight changes

Body weight was monitored during induction of both the periodontitis and arthritis. There were no significant differences in the per cent weight change between the groups during the periodontitis stage (Fig. 1a). Following induction of arthritis there was a greater percentage of weight loss in the two groups that had the CAIA (Fig. 1b). Although, there was a greater weight loss in the group with periodontitis and arthritis compared with arthritis alone from day 4, this was not significant ($p > 0.05$).

Joint inflammation

The macroscopic clinical images of the paws shown in Fig. 2 are representatives of mice within each group at day 8 (post-arthritis induction) when disease was found to be at its maximum. More severe redness and swelling was seen in the front and rear paws of mice with both diseases compared with mice that received only arthritis. It was also more common for mice in the combined periodontitis and arthritis group to demonstrate signs of disease in their rear paws compared with mice with arthritis only.

Paws were scored for signs of redness and swelling as described in the 'Methods' (Fig. 3). In pilot studies (data not shown) paw scores with the 5 μg dose of LPS were not significantly different to those when 10 μg of LPS ($p > 0.05$) was used. With the 5 μg dose at day 3 (LPS injections), all mice with pre-existing periodontitis were showing signs of disease with average scores 3.5 times higher than that of the arthritis alone group. There were significantly higher paw scores for the periodontitis and arthritis group at days 6, 8, 9 and 10 ($p < 0.05$) using a 16-point method of scoring. Similar observations were also made in mice receiving only the CAIA antibody with no LPS. Mice in the group with periodontitis and arthritis (no LPS) similarly to the LPS mice developed arthritis at a greater rate compared with arthritis alone (no LPS). When combining the results of mice pre-existing periodontitis that received arthritis both with and without LPS at day 3 the incidence of disease was 88% compared with 28% in mice with no pre-existing periodontitis.

This observed clinical paw inflammation was also confirmed following assessment of H&E-stained histological sections of mouse front paws (radiocarpal joint) at the completion of the study (Fig. 4). In the arthritis alone group there was evidence of infiltrating polymorphonuclear and mononuclear cells. The inflammation in the periodontitis and arthritis group was more severe with extensive pathological changes seen in the bone and soft tissues. There was evidence of synovial hyperplasia, with significantly high numbers of inflammatory cells. Blinded analysis of the radiocarpal joint in the front paws demonstrated that inflammation in mice with both periodontitis and arthritis was significantly higher than in the periodontitis alone and arthritis alone groups (Fig. 5a).

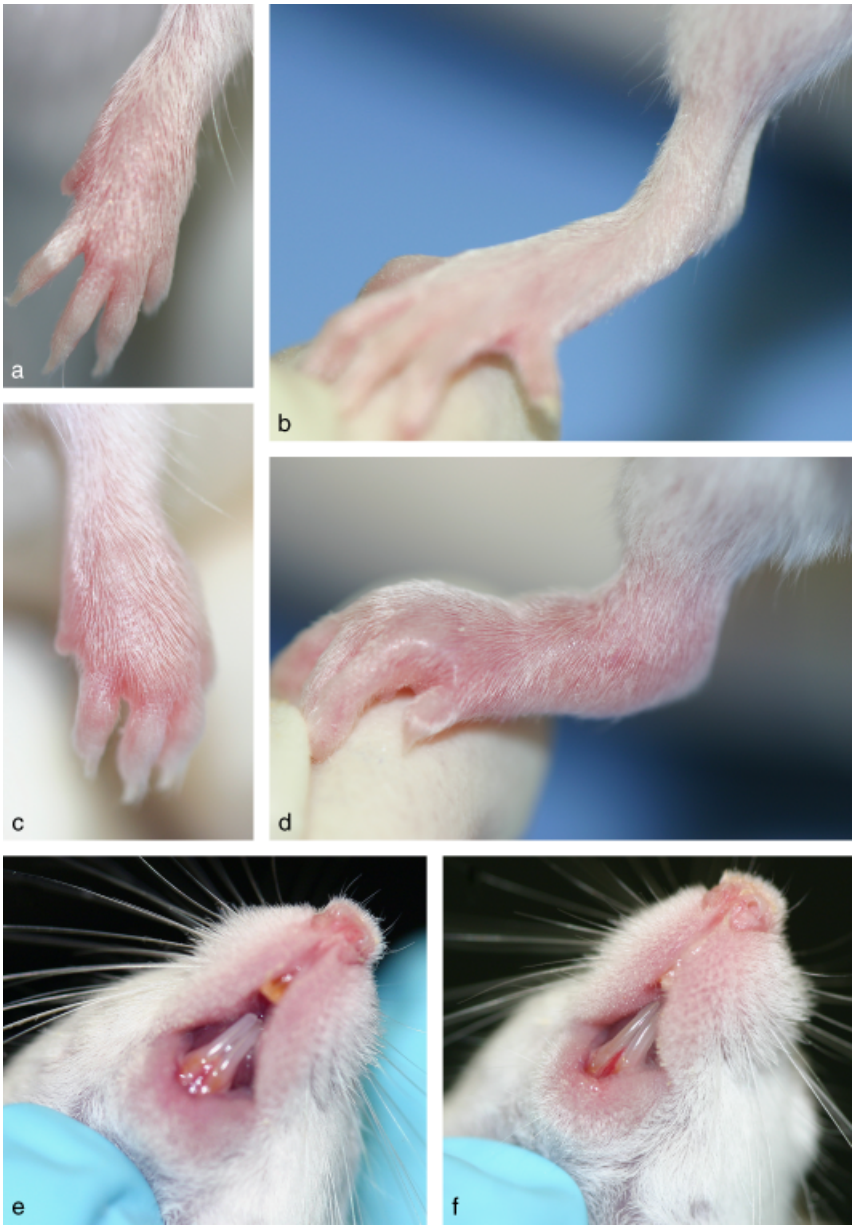

Fig. 2. Macroscopic appearance of the paws and gingival tissues. (a) Experimental arthritis (EA) only front paw; (b) EA only rear paw; (c) periodontitis and EA front paw; (d) periodontitis and EA rear paw; (e) control gingiva; (f) periodontitis gingiva. Paws were imaged at day 8 post arthritis induction.

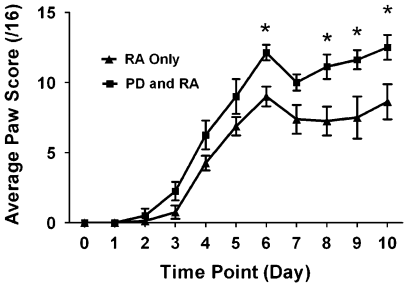

Fig. 3. (a) Average paw scoring using the /16 method as described in the 'Methods' section ($n = 4$ per group). Periodontitis and experimental arthritis (EA) score was significantly higher than RA alone $^{*}p < 0.05$ (b).

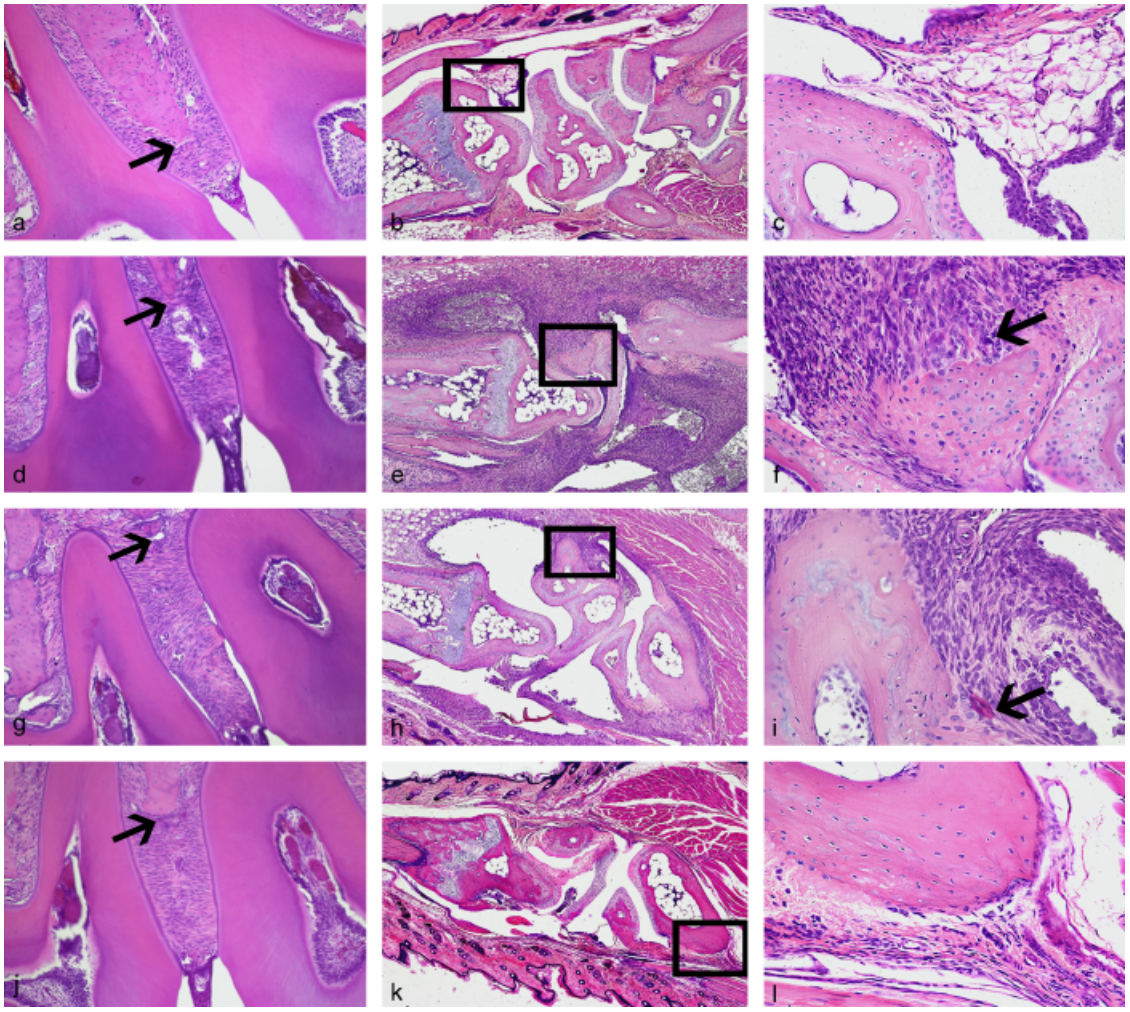

Fig. 4. Histological appearance of periodontal tissues (a,d,g,j) at × 20 magnification and front paw joints (b,e,h,k) at × 4 magnification and (c,f,i,l) at × 20 magnification. (a,b,c) Controls (d,e,f) periodontitis and experimental arthritis (EA) (g,h,i) EA only (j,k,l) periodontitis only. The boxes in the second panel highlight the areas imaged at × 20 magnification in the third panel. Arrows in the first panel indicate level of alveolar bone crest. In the third panel arrows indicate the presence of multinucleated osteoclast cells.

Inflammation in the periodontal tissues

Macroscopic assessment of the incisors indicated that the gingival tissues were red and inflamed and confirmed that a gingival response had been induced (Fig. 2) although, this can be difficult to appreciate due to the nature of this model. The molars, which are the main affected sites, cannot be imaged macroscopically in a longitudinal study of this kind. Micro-CT analysis at the day 44 confirmed alveolar bone loss consistent with our previous studies (Bendyk et al. 2009, Cantley et al. 2009a). The increased CEJ to ABC length at scan 2 also confirms disease induction (Fig. 6). Histological assessment demonstrated a robust inflammatory infiltrate in the periodontitis and combined periodontitis and arthritis groups (Fig. 5). Interestingly, the arthritis alone group manifested some histological evidence of gingival inflammation (Figs 4 and 5). Further analysis and scoring of the periodontal tissues confirmed that inflammation was present in all three of the test groups with the arthritis alone group and combined periodontitis and arthritis groups having greater inflammation than the periodontitis alone (Fig. 5).

Micro-CT analysis of joint and periodontal bone loss

Live animal micro-CT analysis was used to assess changes in BV in the radiocarpal joints of the paws and alveolar bone in the mouth (Fig. 6). From the initial baseline scan to scan 2 (after inducing periodontitis for 36 days) there was an average 16% increase in BV for both front and left-hand side paws as would be expected with the normal growth of these mice. The BV analysis of the front paws indicated that arthritis had been induced with a significant decrease in BV compared with controls (Fig. 6). From scan 2 to scan 3 there was an average 14% decrease in BV for both the arthritis only and combined periodontitis and arthritis groups compared with the normal 18% increase in BV observed in control mice. From scan 2 to 3 there was a decrease in BV in the paws of mice with periodontitis ($p < 0.01$). In the histological sections there was also some evidence of resorption in the periodontitis alone group but little evidence of inflammation (Fig. 4). There was no significant difference in the percentage change in BV from scan 2 to 3 in the arthritis group and the

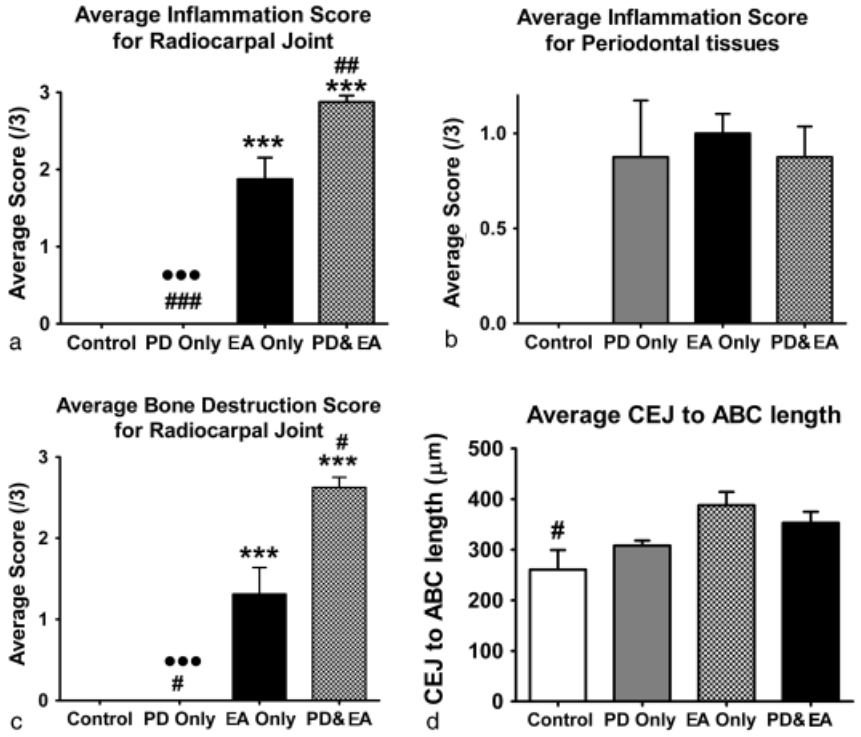

Fig. 5. Average total histological scores for inflammation in (a) the radiocarpal joints (b) periodontal tissues. Bone destruction in (c) radiocarpal joints (d) average cemento-enamel junction to alveolar bone crest length in μm. $^{***}p<0.001$ compared with controls. $\#\#\#p<0.001$. $\#\#p<0.01$. $\#p<0.05$ compared with experimental arthritis (EA) only. $\bullet\bullet\bullet p<0.001$ compared with periodontitis and EA. Details of scoring can be found in the methods.

combined periodontitis and arthritis group, indicating that although there are significant differences in inflammation during this time, the extent of bone destruction is similar. Scoring of histological sections for bone and cartilage destruction demonstrated a significant loss in the group with periodontitis and arthritis compared with arthritis alone (Fig. 5).

Analysis of the alveolar bone surrounding the three maxillary left molars using micro-CT demonstrated that periodontitis had effectively been induced with a 18% decrease in BV from scan 2 (day 0 of RA induction) to scan 3 (10 days after arthritis induction). Overall, in the periodontitis alone group, from the baseline scan to the final scan there was 5% decrease in bone loss, whereas in the group that also received arthritis there was a 23% decrease in BV, indicating enhanced bone destruction in the jaw in mice with both diseases. Interestingly, there was also a 16% decrease in BV of alveolar bone in mice that only received arthritis, which is also evident in the histological image (Fig. 5c). The distance from the CEJ to ABC was increased in mice with periodontitis (Fig. 6). For scan 2 the CEJ–ABC length was significantly higher for both periodontitis groups compared with control confirming disease induction ($p<0.01$). There were no significant differences in CEJ to ABC length for all diseased groups during scan 2. For scan 3 the CEJ to ABC length was significantly higher in the arthritis alone group compared with control confirming the presence of alveolar bone destruction.

RANKL immunohistochemistry

Immunostaining for RANKL demonstrated high levels in the inflamed soft tissue of joints from mice with both periodontitis and arthritis (Figs 7 and 8a). High levels were particularly noted in the pannus region where active bone loss was apparent as would be expected in these groups. Healthy animals and animals with periodontitis alone showed no evidence of pannus in growth into the bone and lacked strong staining in the region where the synovial tissues attached to the bone cartilage interface. RANKL was also detected in the bone-lining cells as well as some chondrocytes and osteocytes of the radiocarpal joint. Similar, but slightly weaker, staining in both the soft tissue and bone was evident in the group with arthritis alone. We did note some weak staining in the synovial tissues of animals with periodontal disease alone and RANKL was only very weakly expressed in the tissues of healthy animals. RANKL was also detected in the periodontal tissues of mice with periodontitis alone and the combined periodontitis and arthritis group. There was mild RANKL staining in the periodontal tissues of the arthritis alone group and weaker staining in healthy tissues (Fig. 8a).

Serum CRP

CRP levels were assessed in serum collected from mice 10 days after arthritis induction. Significantly higher levels of CRP were detected in all groups compared with the control mice. Mice with both periodontitis and arthritis also demonstrated significantly higher CRP levels compared with mice with periodontitis alone and arthritis alone ($p<0.05$) (Fig. 8b).

Discussion

In recent years, a relationship between periodontitis and RA has become increasingly apparent. In a previous study, we established that an extra synovial inflammation, similar to but not identical to periodontitis, exacerbated RA (Bartold et al. 2010a, b). In the present investigation, we have extended these studies to include an assessment of an infective component (*P. gingivalis*-induced periodontitis) to the development of EA. The individual roles of infective viable *P. gingivalis* and its antigens (non-infective) in the interaction between periodontitis and arthritis will now require further study.

The results of this study demonstrate that, in mice, a pre-existing periodontitis significantly influences the induction and severity of CAIA. This observation is consistent with clinical studies in which individuals with periodontitis have more significant RA (Ribeiro et al. 2005, Havemose-Poulsen et al. 2006). The observed exacerbation of arthritis by pre-existing periodontitis could be related to the systemic effects of inflammatory cytokines as a result of the pre-existing chronic inflammation associated with the periodontitis (Bartold et al. 2010a, b). Recently, it has been demonstrated that in AIRmax mice co-induction of periodontal disease

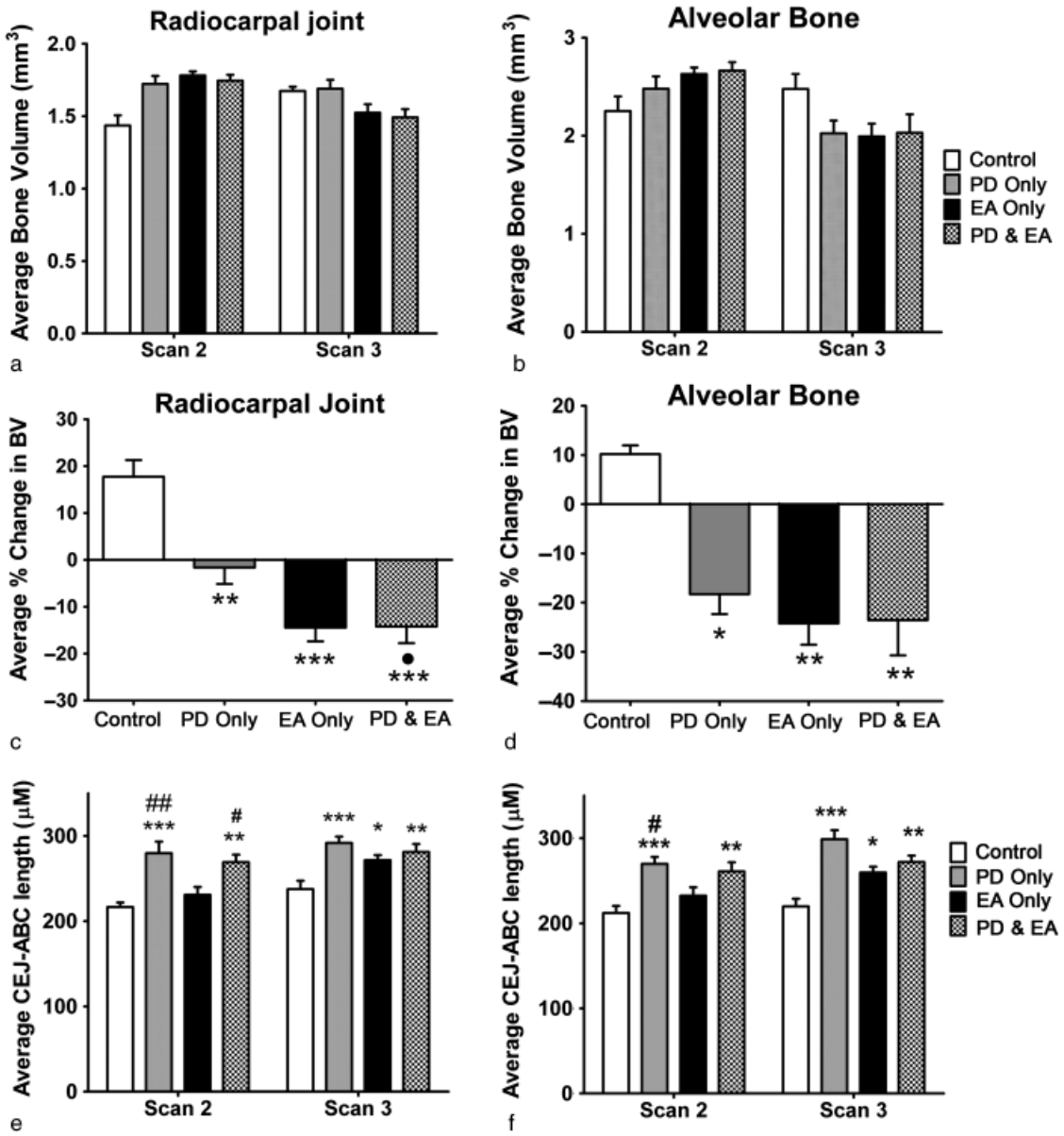

Fig. 6. (a) Absolute bone volume (BV) in mm^3 in the radiocarpal joint as assessed by micro-CT analysis for scan 2 and scan 3 (scan 2 – after inducing periodontitis, before experimental arthritis (EA) induction, scan 3 – final scan). (b) Absolute BV in mm^3 of alveolar bone as assessed by micro-CT analysis for scan 2 and scan 3. (c) Average percentage change in bone volume of the radiocarpal joint during the EA stage as determined using live animal micro-CT scanning. Bars represent mean ± SEM ($n = 4$ per group), (d) average percentage change in bone volume of the alveolar bone supporting the three molars of the maxilla on the left-hand side. Bars represent mean ± SEM ($n = 4$ per group), (e) average length of cemento-enamel junction (CEJ) to alveolar bone crest (ABC) (μm) between the first and second molars. (f) Average length of CEJ to ABC (μm) between the second and third molars. $^{***}p < 0.001$, $^{**}p < 0.01$ $^{*}p < 0.05$ compared with control. • $p < 0.05$ compared with periodontitis only. ##$p < 0.001$, #$p < 0.05$ compared with RA only.

and arthritis, using the model of PIA, resulted in higher serum levels of IL-1β, IFN-γ, IL-17, RANKL and MMP-13 levels compared with the PIA alone (Trombone et al. 2010). It has been proposed that these inter-relationships may be related to dysregulation of the inflammatory system and a systemic increase in pro-inflammatory cytokines (Mercado et al. 2003, Bartold et al. 2005, 2010a, b) and a general systemic inflammation. The elevated RANKL expression in joint and periodontal tissues and increased serum CRP levels in the test groups of the present study lend support to the effect of systemic inflammation on both periodontitis and RA.

The above findings are in contrast to another study, which investigated the co-induction of arthritis and periodontitis in AIRmin mice (Trombone et al. 2010). This could be due to the differences in experimental arthritis models in which different severities of arthritis induction could be related to the development of a pre-existing periodontitis. Furthermore the sequence of disease induction differed between these two studies. In our study, periodontitis was induced before the induction of arthritis. The reasoning behind developing a pre-existing periodontitis is related to the clinical situation. Clinical investigations by our group have suggested that patients with severe RA are more likely to have advanced periodontitis and vice versa

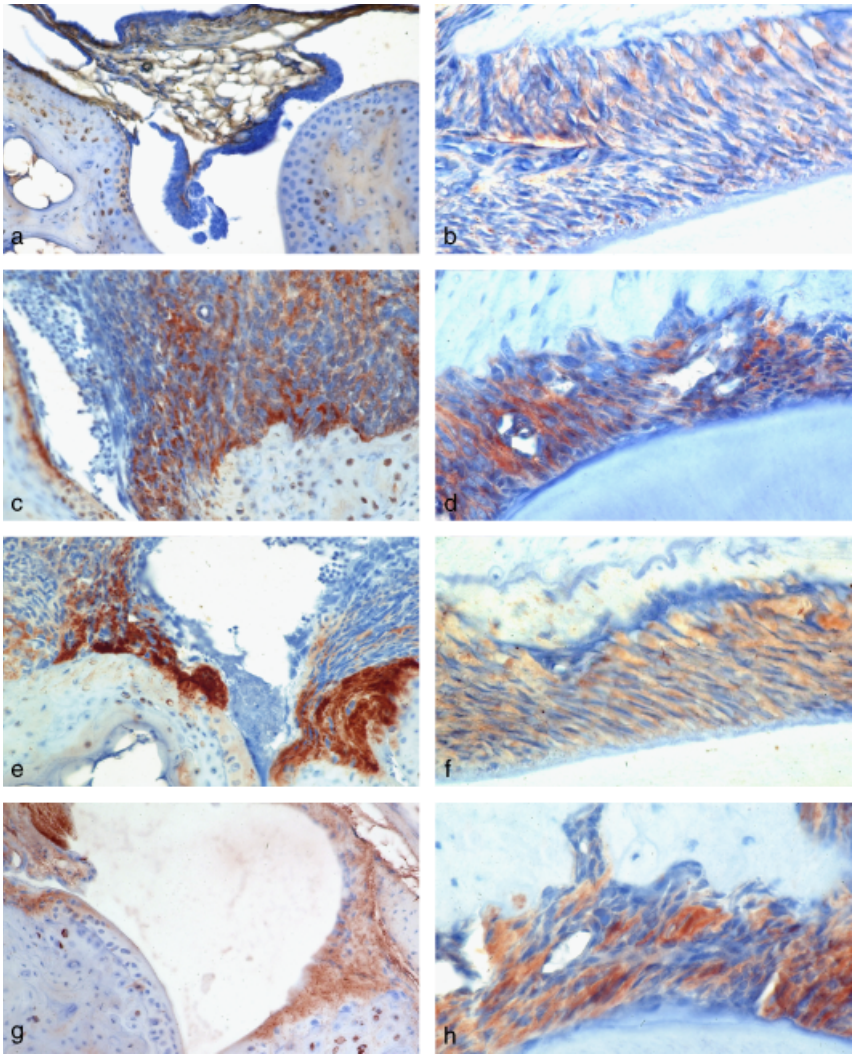

Fig. 7. Immunostaining to detect RANKL in the radiocarpal joints (a,c,e,g) and periodontal tissues (b,d,f,h). Controls (a and b), periodontitis and experimental arthritis (EA) (c and d); EA only (e and f); periodontitis only (g and h). Original magnification × 20. In the periodontal tissue images alveolar bone is at the top of the section and the tooth at the bottom.

(Mercado et al. 2000, 2001). It has also been noted that patients with RA taking anti-inflammatory medications, which are likely to reduce periodontal inflammation, still have signs of periodontal destruction indicating that periodontitis may develop early and is not detected before development of RA (Bartold et al. 2005).

In this model of CAIA, we used a lower dose of LPS, 5 μg compared with 10 μg used in previous studies. This lower dose was used to induce a noticeable arthritic response that allows us to assess the effect of pre-existing periodontitis on the response. There were no significant differences in paw scoring observed between mice given the lower 5 μg of LPS compared with 10 μg. The major difference in disease severity with the different doses included the rear paws more commonly being affected in the higher LPS group. This did not impact the results as the front paws were used for CT and histological analysis. It was interesting to note that rear paws of mice with periodontitis and arthritis were more commonly affected.

Despite significantly higher levels of inflammation in the radiocarpal joints in mice with both periodontitis and arthritis compared with arthritis alone, there was no significant difference in bone loss as detected by CT analysis. Although scoring of histological bone and cartilage destruction did demonstrate significant levels of higher bone loss in the periodontitis and arthritis group compared with arthritis alone. This could be due to methodology differences and a later time point CT scan may demonstrate differences but this was not conducted due to ethics.

In addition to noting the effect of pre-existing periodontitis on the development of arthritis, we found evidence of bone destruction and increased RANKL expression in the radiocarpal joints of mice that had periodontitis only. This finding further highlights the possibility that extra-synovial infection and/or inflammation has the potential to influence the synovial tissues (Bartold et al. 2010a, b). The mechanisms underlying this finding remain to be elucidated. Because there was no overt inflammation noted in the synovial tissues of mice with periodontitis alone, the mechanisms for these joint changes in these animals remains to be established. Nonetheless, the observation that RANKL expression was elevated in the joints of these animals indicates that some systemic influences have the potential to affect local sites. Such findings have been noted in RA whereby generalized osteopenia is noted and is thought to arise through the systemic action of inflammatory cytokines released from the site of synovial inflammation (Deodhar & Woolf 1996, Guler-Yuksel et al. 2008, 2009, Goldring 2009).

The converse finding from this study is that mice with arthritis alone showed signs of alveolar bone destruction implying that experimental arthritis may increase the severity of periodontitis. CT analysis revealed evidence of a BV loss in mice with arthritis only and a significantly longer CEJ to ABC length compared with controls as assessed by CT and histological sections. While this could not be directly assessed in this study due to the timing of disease induction, this is also a possibility. Similar findings of periodontal changes being observed following the induction of adjuvant arthritis in rats have been reported (Ramamurthy et al. 2005, Park et al. 2010). In our model, the periodontal changes observed in the arthritis only animals may be related to the LPS dosing given for the induction of CAIA, which was introduced as an i.p. injection, and thus entered the systemic circulation with the potential to have effects at sites throughout the body. However, the mechanisms through which these changes may arise are unclear and warrant further investigation. Nonetheless, the result of periodontal bone destruction observed in mice with arthritis is consistent with

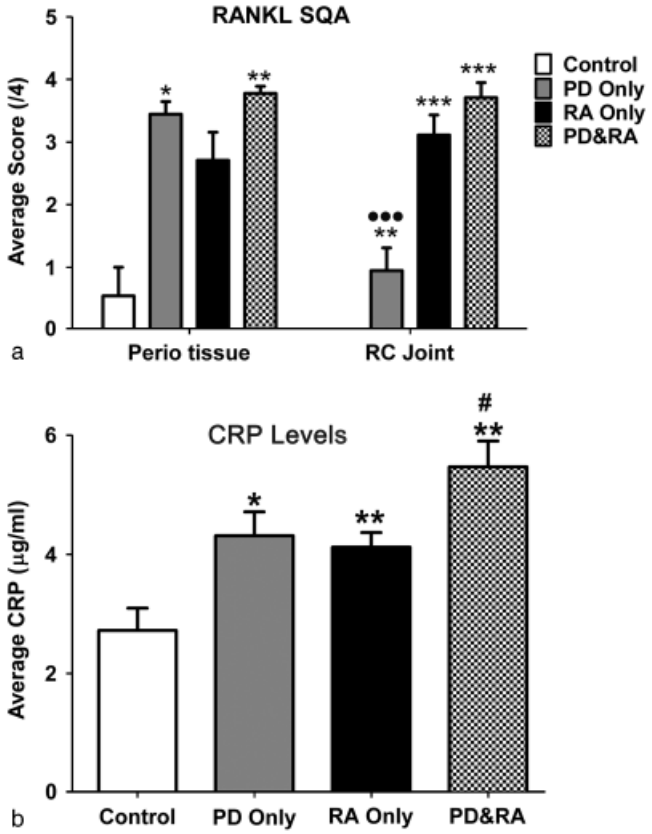

Fig. 8. (a) Semiquantitaive analysis (SQA) of RANKL positive cells for radiocarpal joints and periodontal tissues. (b) Average serum C-reactive protein (CRP) levels (µg/ml) measured using an ELISA. $^{***}p<0.001$ $^{**}p<0.01$ and $^{*}p<0.05$ compared with control. $\#p<0.05$ compared with EA only. • • • $p<0.001$ compared with periodontitis and EA group.

clinical studies that have demonstrated significantly higher incidences of tooth loss and alveolar bone loss in patients with RA (Mercado et al. 2000, 2001, Al-Shammari et al. 2005).

A number of mechanisms which may account for a relationship between periodontitis and RA have been proposed (Bartold et al. 2005). These may include vascular alterations and osseous changes related to altered RANKL/osteoprotegerin ratios, bacterial infection and enhancement of autoimmunity through citrullination of proteins. Of these, autoimmunity characterized by production of antibodies against citrullinated proteins is of particular interest because *P. gingivalis* produces deimination enzymes such as peptidyl arginine deaminase and this may lead to citrullination of proteins within the periodontal tissues. Accordingly, the potential for citrullination of proteins by *P. gingivalis* and subsequent generation of autoantigens, which could drive autoimmunity in RA has been proposed as one mechanism linking periodontitis and RA (Rosenstein et al. 2004, Lundberg et al. 2010).

In conclusion the key finding of this study indicates that pre-existing periodontitis exacerbated arthritis in a mouse model. While statistically significant, this relationship was modest. Furthermore, there is some potential that a bidirectional relationship between periodontitis and arthritis may exist. That is, not only can periodontitis influence the joint tissues but arthritis can also influence the periodontal tissues. Future studies will need to address the mechanisms through which these associations act and the model developed in this study would provide a valuable tool for such studies. In addition, this model could be used to investigate whether treatment of one condition can have any benefit on the other as well as test new therapeutic targets to determine effects on both disease states.

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Clinical Relevance

Scientific rationale for study: Previous studies have indicated a significant relationship between RA and periodontitis. In this study, we have extended our previous animal model of periodontal disease to develop an animal model in which pre-existing periodontitis is established in mice followed by induction of RA to investigate the relationship between these two prevalent conditions.
Principal findings: Pre-existing periodontitis induced by *P. gingivalis* exacerbated the onset and severity of experimental RA.
Practical implications: Control of periodontal infection and inflammation may be important in the management of RA.