Periodontal Pathogens Directly Promote Autoimmune Experimental Arthritis by Inducing a TLR2- and IL-1-Driven Th17 Response

Sabrina G. de Aquino, Shahla Abdollahi-Roodsaz, Marije I. Koenders, Fons A. J. van de Loo, Ger J. M. Pruijn, Renoud J. Marijnissen, Birgitte Walgreen, Monique M. Helsen, Liduine A. van den Bersselaar, Rafael S. de Molon, Mario J. Avila Campos, Fernando Q. Cunha, Joni A. Cirelli and Wim B. van den Berg

J Immunol 2014; 192:4103-4111; Prepublished online 28 March 2014;
doi: 10.4049/jimmunol.1301970
http://www.jimmunol.org/content/192/9/4103

Supplementary Material http://www.jimmunol.org/content/suppl/2014/03/30/jimmunol.1301970.DCSupplemental.html

References This article cites 52 articles, 8 of which you can access for free at:
http://www.jimmunol.org/content/192/9/4103.full#ref-list-1

Subscriptions Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscriptions

Permissions Submit copyright permission requests at:
http://www.aai.org/ji/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/cgi/alerts/etoc
Periodontal Pathogens Directly Promote Autoimmune Experimental Arthritis by Inducing a TLR2- and IL-1–Driven Th17 Response

Sabrina G. de Aquino,*†,1 Shahla Abdollahi-Roodsaz,*†,1 Marijke I. Koenders,*
Fons A. J. van de Loo,* Ger J. M. Pruijn,‡ Renoud J. Marijnissen,* Birgitte Walgreen,*
Monique M. Helsen,* Liduine A. van den Bersselaar,* Rafael S. de Molon,†
Mario J. Avila Campos,§ Fernando Q. Cunha,§ Joni A. Cirelli, † and Wim B. van den Berg*

Increasing epidemiologic evidence supports a link between periodontitis and rheumatoid arthritis. The actual involvement of periodontitis in the pathogenesis of rheumatoid arthritis and the underlying mechanisms remain, however, poorly understood. We investigated the influence of concomitant periodontal pathologies on clinical and histopathologic characteristics of T cell–mediated experimental arthritis and evaluated modulation of type II collagen (CII)–reactive Th cell phenotype as a potential mechanism. Repeated oral inoculations of periodontal pathogens Porphyromonas gingivalis and Prevotella nigrescens induced periodontitis in mice, as evidenced by alveolar bone resorption. Interestingly, concurrent periodontitis induced by both bacteria significantly aggravated the severity of collagen-induced arthritis. Exacerbation of arthritis was characterized by increased arthritic bone erosion, whereas cartilage damage remained unaffected. Both P. gingivalis and P. nigrescens skewed the CII-specific T cell response in lymph nodes draining arthritic joints toward the Th17 phenotype without affecting Th1. Importantly, the levels of IL-17 induced by periodontal pathogens in CII-specific T cells directly correlated with the intensity of arthritic bone erosion, suggesting relevance in pathology. Furthermore, IL-17 production was significantly correlated with periodontal disease–induced IL-6 in lymph node cell cultures. The effects of the two bacteria diverged in that P. nigrescens, in contrast to P. gingivalis, suppressed the joint-protective type 2 cytokines, including IL-4. Further in vitro studies showed that the Th17 induction strongly depended on TLR2 expression on APCs and was highly promoted by IL-1. Our data provide evidence of the involvement of periodontitis in the pathogenesis of T cell–driven arthritis through induction of Ag-specific Th17 response.


Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation in multiple joints. Although the exact etiology of RA remains unknown to date, production of proinflammatory cytokines and activation of lymphocytes are considered as crucial events in disease pathogenesis (1, 2). In this line, the Th17 lymphocytes and IL-17 have been recognized as essential mediators of cartilage and bone destruction (2–4). The number of Th17 cells is increased in the early stages of the disease and in active RA (5, 6). However, the factors promoting the Th17 differentiation in RA are poorly understood.

In light of the critical role of innate immunity in instructing the T cell response and the increasing evidence for the involvement of TLRs in RA, normal as well as infectious microbiota have recently emerged as triggers for RA (7). In this context, the influence of microbial environment on arthritis was shown in several Th17-driven mouse models, where the spontaneous disease in mice with T cell–activating genetic modifications was abrogated under germ-free or specific pathogen-free conditions (8–10). Furthermore, the development of arthritis is affected by TLR2 and TLR4 activation, which can influence the T cell balance in arthritic mice in vivo (8, 11).

In addition to normal microbiota, infectious agents, including periodontal pathogens, have been increasingly implicated in the etiology of RA (12, 13). The link between periodontal disease (PD) and RA has primarily been based on the shared pathophysiology, characterized by destructive inflammation, and is supported by growing epidemiologic evidence (14). In this respect, case control studies have verified both higher prevalence as well as presence of more severe forms of PD in RA patients (13, 14). Other studies showed a reduction of the severity of active RA when the accompanying PD was successfully treated (15, 16).
addition, enhanced Ab titers against Porphyromonas gingivalis, the major causative agent of periodontitis, have been detected in serum of patients with RA as well as in individuals at increased risk of developing RA (17, 18).

Despite the vast amount of epidemiologic evidence linking PD and RA, the actual implications of periodontitis in the pathophysiology of RA and the underlying mechanisms remain poorly understood. Based on the significant role of pathogen-mediated TLR activation in shaping the T cell response, we reasoned that the interaction between PD and RA may in part be a direct result of skewing the Th cell balance. The aim of the current study was to investigate the influence of periodontitis on clinical severity and specific histopathologic features of T cell–dependent experimental arthritis with particular focus on disease-relevant Th cell phenotype. Two periodontal bacteria, P. gingivalis and Prevotella nigrescens, of which the DNA has been detected in RA serum and synovial fluid (19, 20), were compared. The data reveal exacerbation of autoimmune arthritis by periodontitis induced by both bacteria through induction of collagen-specific Th17 phenotype directly correlated with arthritic bone erosion, along with suppression of the joint-protective IL-4 by P. nigrescens in particular. The Th17 induction by both bacteria was found dependent on TLR2 activation on APCs, involved IL-1, and was correlated with IL-6 production.

Materials and Methods
Induction of collagen-induced arthritis and periodontitis
Bovine type II collagen (CII) was dissolved in 0.05 M acetic acid to a concentration of 0.5 mg/ml and emulsified in an equal volume of CFA containing 0.5 mg/ml Mycobacterium tuberculosis H37Ra (Difco Laboratories). Male 10- to 12-wk-old DBA-1 mice (Janvier) were immunized by intradermal injection of 100 μl of the emulsion at the tail base. Periodontitis was induced in collagen-induced arthritis (CIA) mice by five oral inoculations of 1 × 10^8 CFU P. gingivalis ATCC 33277 and P. nigrescens ATCC 33563 anaerobically grown on supplemented blood agar. Bacteria were diluted in 75 μl PBS containing 2% carboxymethylcellulose and inoculated repeatedly on alternate days starting from day 14 to induce PD. Control group received 2% carboxymethylcellulose alone. In control studies, 10^9 CFU periodontal bacteria were heat killed (30 min at 60˚C) and injected i.p. together with 50 μg CII on day 21 of initial CIA induction. All protocols were approved by the Animal Ethics Review Board of Radboud University Nijmegen. All experiments were performed according to the codes of practice defined by the Dutch law on animal experimentation (article 18a).

Assessment of arthritis
Arthritis was macroscopically scored by two blinded observers on a 0–2 scale per paw, as described previously (21). For histological analysis, ankle joints isolated at day 30 were fixed in 4% formaldehyde for 4 d, then decalcified in 5% formic acid, and embedded in paraffin. Tissue sections of 7 μm were stained with H&E to study synovial inflammation or with safranin O to determine cartilage and bone destruction. Each parameter was scored on a scale from 0 to 3 by two independent observers in a blinded manner.

Microcomputed tomography scanning and alveolar bone loss analysis
Periodontitis was assessed by evaluating the alveolar bone loss using a microcomputed tomography (μCT) technique. To this end, maxillae were harvested on day 30, immediately fixed in 10% formalin for 24 h, and transferred to 70% alcohol until the scanning procedure. Maxillary samples were scanned and reconstructed at 18 μm voxels using a μCT system (Skyscan, Aartselaar). Reconstruction of three-dimensional images was followed by spatial reorientation of sagittal plane using NRecon and DataViewer softwares (Skyscan, Aartselaar). Anatomical references and standard dimensions were defined for bone loss analysis using CTAn software (Skyscan, Aartselaar), as previously described (22). In brief, two-dimensional regions of interest were defined as the distance from the cementoenamel junction to a fixed base (3 mm in the apical direction) at the palatal surface of the roots of the first and second molar teeth. The two-dimensional regions of interest were drawn at regular intervals on a sagittal view and reconstructed as a three-dimensional form to quantify volumetric parameters. Bone volume fraction data were used to compare the bone loss among the experimental groups.

Generation and stimulation of bone marrow–derived dendritic cells
Bone marrow cells were isolated from 5- to 6-wk-old BALB/c and cultured for 9 d with 20 ng/ml GM-CSF (R&D Systems). Dendritic cell (DC) phenotype was controlled by FACS using anti-CD11c allophycocyanin (Abs (BD Pharmingen)). Cytokine response to periodontal pathogens was assessed in 1 × 10^6 DCs incubated with 1 μg/ml Pam3Cys and heat-killed (30 min at 60°C) P. gingivalis or P. nigrescens (10^5 CFU/well) for 24 h.

Culture and analysis of T lymphocytes
To analyze the T cell response during CIA, cells from popliteal and inguinal lymph nodes (day 30) were pooled and cultured (2 × 10^6 cells/well) in the presence of plate-coated anti-CD3 (1 μg/ml; R&D Systems) and soluble anti-CD28 (2 μg/ml; BD Biosciences), or bovine CII (50 μg/ml) for 96 h.

For in vitro analysis of Th cell differentiation by periodontal bacteria, splenic CD4+ T cells were isolated from nonarthritic BALB/c, IL-1R antagonist (IL-1Ra)–/–, and, where mentioned, IL-1Ra–/–/TLR2–/– mice using MACS (Miltenyi Biotec). CD4+ T cells (50,000/well) and 30 Gy-irradiated CD4+ cells (150,000/well as APCs) were cocultured in the presence of plate-coated anti-CD3 (1 μg/ml; R&D Systems), and either Pam3Cys (1 μg/ml; EMRC Microcollections), heat-killed P. gingivalis, or P. nigrescens (10^5 CFU/well each) for 72 h. Culture supernatant was collected for measurement of cytokines. For flow cytometry, cells were harvested and stimulated with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (1 μg/ml; Sigma-Aldrich), and the Golgi-traffic inhibitor brefeldin (1 μl/ml; BD Biosciences) for an additional 4 h. T cell differentiation was determined using anti-CD4 allophycocyanin, anti-IL–17 PE, and anti–IFN-γ FITC staining and appropriate isotype-matched control Abs (all from BD Biosciences-Pharmingen). Cells were analyzed on a FACSCalibur using the CellQuest software (BD Biosciences-Pharmingen).

Measurement of cytokines
Cytokine and chemokine concentrations in culture supernatants and mice sera were determined using the Milliplex cytokine assays from Millipore following the manufacturer’s instruction.

Isolation of RNA from synovial biopsies
Synovial biopsies from knee joints were isolated from lateral and medial sides of patellae using a 3-mm skin biopsy punch (Stiefel) at day 30 of CIA. RNA isolation, quantitative PCR analysis, and GAPDH and cathepsin K primer sequences were as described before (23).

Statistical analysis
Group measures are expressed as mean ± SEM. Statistical significance was assessed using the two-tailed Mann–Whitney U test to compare two experimental groups and the Kruskal–Wallis test to compare more than two groups. Correlations were examined using Spearman’s rank test. Analyses were performed on GraphPad Prism 4.0 software (GraphPad Software).

Results
Periodontitis induced by P. gingivalis and P. nigrescens increases the severity of experimental arthritis
To examine whether induction of periodontitis affects the progression of experimental arthritis in vivo, mice immunized with CII received five oral inoculations of either P. gingivalis or P. nigrescens every other day, as described in Materials and Methods. Repeated oral inoculations of both bacteria induced signs of periodontitis, as evidenced by alveolar bone loss observed in μCT analysis of the maxillae (Fig. 1A, 1B). Interestingly, periodontitis induced by P. gingivalis and P. nigrescens accelerated the onset of arthritis. Moreover, shortly after the last bacterial inoculation (day 22), arthritis incidence was higher in bacteria-treated groups (70% for both P. gingivalis and P. nigrescens groups) compared with the control group (40%). The disease
incidence at the end point (day 30) was similar, that is, 60% for control mice, 70% for *P. gingivalis*, and 80% for *P. nigrescens* groups. Importantly, coincident periodontitis induced by *P. gingivalis* significantly increased the clinical severity scores of arthritis, and a similar tendency was observed for *P. nigrescens*-induced periodontitis as well (Fig. 1C). In contrast, systemic injection of heat-killed *P. g.* or *P. n.* together with CII booster on day 21 of CIA does not affect arthritis severity. (E and F) Increased serum levels of IL-6 (E) and keratinocyte-derived chemokine (KC) (F) at day 30 of arthritis with concomitant periodontitis, as shown in (C), measured by Milliplex. Data are shown as mean ± SEM of 9–10 mice per group. *p < 0.05*, **p < 0.01 versus control by the Mann–Whitney U test. n.s., not significant.

**FIGURE 1.** Concurrent periodontitis exacerbates experimental arthritis and induces proinflammatory cytokines. Mice with CIA received five oral inoculations of $10^6$ living *P. gingivalis* (*P.g.*) or *P. nigrescens* (*P.n.*) starting at day 14 after immunization with CII without additional CII booster. (A) Evidence of periodontitis shown by alveolar bone loss on microcomputed tomography analysis of the maxillae isolated on day 30 after immunization with collagen. (B) Representative bidimensional and threedimensional sagittal microcomputed tomography views of the maxillary molars from each group showing alveolar bone loss. (C) Increased clinical scores of arthritis in the fore and hind paws upon concomitant periodontitis. (D) Intraperitoneal injection of $10^{10}$ heat-killed *P.g.* or *P.n.* together with CII booster on day 21 of CIA does not affect arthritis severity. (E and F) Increased serum levels of IL-6 (E) and keratinocyte-derived chemokine (KC) (F) at day 30 of arthritis with concomitant periodontitis, as shown in (C), measured by Milliplex. Data are shown as mean ± SEM of 9–10 mice per group. *p < 0.05*, **p < 0.01 versus control by the Mann–Whitney U test. n.s., not significant.

Coincident periodontitis significantly increases the severity of bone erosion in arthritic joints

To determine which specific histopathological features of arthritis are affected by periodontitis, ankle joint sections were analyzed microscopically. Analysis showed a tendency toward increased synovial inflammation, that is, both exudate and infiltrate, upon oral inoculation of periodontal pathogens; however, statistical significance was not reached (*p = 0.22* for *P. gingivalis* and *p = 0.35* for *P. nigrescens* compared with control; Fig. 2A). Furthermore, no apparent effect on the severity of cartilage destruction was observed (Fig. 2B). Interestingly, the severity of arthritic bone erosion was substantially enhanced by *P. gingivalis* as well as *P. nigrescens*-induced periodontitis (Fig. 2C). In addition, synovial tissue of mice with periodontitis having more severe arthritic bone erosion expressed higher mRNA expression of cathepsin K, an enzyme involved in osteoclastic bone resorption (relative mRNA expression of 22.9 ± 7.6 in control group, 108.9 ± 34.0 in *P. gingivalis* group, and 44.7 ± 8.7 in *P. nigrescens* group; *p < 0.05* by Kruskal-Wallis test). Representative histological images illustrating increased bone erosion in the ankle joint of mice with periodontitis are shown in Fig. 2D.

**Periodontal pathogens specifically induce an IL-17 phenotype in CII-specific T cells while reducing Th2 cytokines**

To understand the mechanism of arthritis exacerbation by coinciding periodontitis, we studied the influence of periodontitis on T cell phenotype in draining (popliteal and inguinal) lymph nodes. Upon pan T cell stimulation using anti-CD3 and anti-CD28 Abs, increased IL-17 production was found in mice inoculated with *P. gingivalis* and *P. nigrescens* compared with the control group (Fig. 3A). IFN-γ levels remained, however, unaffected (Fig. 3B). Furthermore, we did not observe any evidence of the induction of IFN-γ/IL-17 double-positive CD4 T cells. Interestingly, periodontitis specifically induced by *P. nigrescens* resulted in strong suppression of Th2 cytokines IL-4, IL-5, and IL-9 in cells from joint-draining lymph nodes (Fig. 3C–E). These data indicate that both periodontal pathogens are capable of favoring Th17 differentiation in vivo, whereas *P. nigrescens* can reduce the anti-inflammatory Th2 response as well. Importantly, stimulation of T cells with the joint Ag CII revealed that both *P. gingivalis* and *P. nigrescens* significantly increased IL-17 production without influencing IFN-γ (Fig. 3F, 3G), indicating a shift toward the Th17 phenotype in Ag-specific T cells. Moreover, the concentrations of IL-17 produced by Ag-specific T cells significantly correlated with the intensity of bone erosion within the joints, suggesting
relevance for the observed pathology (Fig. 3H). IL-4 levels were very low upon stimulation with collagen (<4 pg/ml, data not shown), suggesting that Ag-specific T cells are not of Th2 type. Furthermore, IL-6 as a critical Th17-polarizing cytokine was found to be increased in lymph node cell cultures from mice with PD (Fig. 3I). Interestingly, IL-6 concentrations were significantly correlated with IL-17 levels (Fig. 3J), suggesting a potential role for IL-6 induction by periodontal pathogens in driving the Th17 differentiation.

Analysis of synovial cytokine expression revealed that the presence of concurrent PD during CIA induced a number of proinflammatory cytokines, along with upregulation of IFN-γ, Tbet (only for P. gingivalis), IL-17A, and RORγt (Supplemental Fig. 1). Although statistical significance was not reached in some cases, simultaneous upregulation of multiple cytokines relevant in Th17 phenotype, as reflected in the draining lymph nodes.

P. gingivalis and P. nigrescens activate DCs to produce T cells affecting proinflammatory cytokines

Next, we examined whether periodontal pathogens would stimulate innate APCs to produce cytokines affecting T cell differentiation, thereby also explaining the divergent effects on Th2 suppression. To this end, bone marrow–derived DCs (BMDCs) were stimulated with P. gingivalis and P. nigrescens along with the TLR2 agonist Pam3Cys as an internal control. Comparison of the production of various cytokines induced by the two bacteria revealed that P. gingivalis and P. nigrescens have similar capability to induce IL-1β and IL-6 (Fig. 4A, 4B) and that P. gingivalis is rather a stronger inducer of IL-12, TNF-α, and, notably, also IL-10 (Fig. 4C–E). IL-5, IL-13, and IL-23 levels were below the detection limit of 2 pg/ml.

P. gingivalis and P. nigrescens strongly induce Th17 differentiation in vitro, an effect highly promoted in the absence of IL-1Ra

Considering the observed in vivo effects on T cell cytokine profiles during arthritis, we investigated the direct influence of periodontal pathogens on T cell differentiation in vitro. Stimulation with heat-killed P. gingivalis and P. nigrescens induced Th1 as well as Th17 differentiation in a coculture of APCs with CD4+ T cells, as measured by flow cytometry (Fig. 5A–C). The percentage of Th17 cells was significantly higher than that of the Th1, suggesting skewing toward Th17 (Fig. 5A, 5B). Furthermore, Th17 induction by periodontal bacteria was strongly promoted in the absence of endogenous IL-1Ra in the culture (~10-fold increase in Fig. 5C compared with 5B). This indicated the prominent involvement of IL-1 in Th17 skewing by both periodontal bacteria in line with the general important role of IL-1 in Th17 differentiation. When using IL-1Ra−/− cells, P. gingivalis and particularly P. nigrescens were also able to induce very low percentages of IFN-γ/IL-17 double-positive CD4+ T cells (data not shown).

In accordance with flow cytometry data, IL-17 production by CD3-triggered T cells was considerably enhanced when the cocultured APCs were stimulated with either P. gingivalis or P. nigrescens and was further aggravated in the absence of IL-1Ra (Fig. 5D, 5E). Both pathogens were relatively weak inducers of Th1 differentiation and IFN-γ production (Fig. 5A–E). IL-1Ra deficiency resulted in an increased Th1 differentiation and IFN-γ production as well; however, the final Th cell response to periodontal bacteria remained far more in favor of Th17 (Fig. 5A–E). Interestingly, cocultures of IL-1Ra−/− APCs and T cells also contained higher concentrations of IL-4 when compared with wild-type cocultures (Fig. 5F). Importantly, stimulation with P. nigrescens significantly reduced IL-4 production (Fig. 5F), an effect in line with the in vivo suppression of the Th2 response upon P. nigrescens-induced PD (Fig. 3C–E).

Th17 induction by P. gingivalis and P. nigrescens is dependent on TLR2 expression on APCs

P. gingivalis has been shown to activate innate immune cells mainly through TLR2 (24, 25). We investigated the involvement of TLR2 in Th cell differentiation induced by P. gingivalis and P. nigrescens using IL-1Ra and IL-1Ra/TLR2 double-deficient

![FIGURE 2.](https://example.com/figure2.png) Concomitant periodontitis aggravates arthritic bone erosion without influencing cartilage pathology. (A–C) Histological assessment of the ankle joints at day 30 after immunization with CII. Joint inflammation (A) was scored on H&E-stained tissue sections. Cartilage destruction (B) and bone erosion (C) were scored on safranin O-stained sections. Data are mean ± SEM (scale 0–3) of 9–10 mice per group. *p < 0.05 versus control by the Mann–Whitney U test. (D) Representative images of safranin O-stained ankle joint sections illustrating increased arthritic bone erosion by periodontal pathogens. Original magnification ×100 in the upper panel and ×200 in the lower panel. P. g., P. gingivalis; P. n., P. nigrescens.)
APCs and T cells. Th17 induction by both bacteria was found to highly depend on TLR2 expression on APCs, as Th17% was markedly reduced when only APCs or both APCs and T cells were TLR2 deficient (Fig. 5G). TLR2 deficiency only on T cells resulted in increased Th17 differentiation (Fig. 5G). In contrast to Th17, the induction of Th1 seemed partially dependent on TLR2 expression directly on T cells rather than on APCs (Fig. 5H). These data point toward a cell-specific involvement of TLR2 in driving the differentiation of either Th1 or Th17 subset upon recognition of periodontal bacteria.

Altogether, these data suggest a role for *P. gingivalis* as well as *P. nigrescens*-induced periodontitis in promoting arthritic bone erosion probably via a TLR2-mediated induction of IL-1 and IL-6 in APCs to induce Th17 phenotype in CIA-reactive T cells. *P. nigrescens* appeared to employ an additional mechanism, that is, suppression of the Th2 response, as well. Modulation of the Th cell phenotype and cytokine production by *P. gingivalis* and *P. nigrescens* as a potential mechanism promoting arthritic bone erosion, in addition to the previously described induction of anti-citrullinated peptide Abs (ACPA) by *P. gingivalis* (17, 26–28), is schematically illustrated in Fig. 6.

**FIGURE 3.** Periodontal pathogens promote Th17, but not Th1, differentiation during arthritis, and *P. nigrescens*, but not *P. gingivalis*, suppresses T cell IL-4 production in vivo. (A–G) Lymph node T cells from CIA arthritic mice of control, *P. gingivalis*- , and *P. nigrescens*-treated groups were stimulated with either anti-CD3 + anti-CD28 Abs (A–E) or CII as the joint-specific Ag (F, G) for 72 h. T cell phenotype was assessed by measuring the prototypic Th cytokines in culture supernatants using Milliplex cytokine array. n = 9–10 mice per group. *p < 0.05, ***p < 0.001 by the Mann–Whitney U test. (H) Correlation between the intensity of arthritic bone erosion and IL-17 production by lymph node T lymphocytes stimulated with CII. (I) IL-6 production by lymph node cells stimulated with anti-CD3 + anti-CD28 Abs for 72 h and (J) correlation between IL-6 and IL-17 production in the same culture. The Spearman’s correlation coefficient (r) and the p value in (H) and (J) are shown at the top right of these figures.
increases the severity of passive, T cell–independent arthritis induced by Abs directed against collagen (30). However, no mechanistic insight was provided in these studies.

In the current study, we sought to examine the presence of a functional pathologic interaction between \textit{P. gingivalis}-and \textit{P. nigrescens}-induced periodontitis and the concomitant autoimmune arthritis. Furthermore, we aimed to assess whether this interaction might occur by modulating the differentiation of Ag-reactive T cells during CIA and studied the underlying TLR and cytokine dependence of Th differentiation induced by periodontal pathogens.

Previous studies on the relationship between RA and PD have primarily focused on \textit{P. gingivalis}, probably because of its ability to citrullinate proteins and generate potential autoantigens (31, 32). However, \textit{P. gingivalis} possesses several other virulence factors such as LPS, fimbriae hemagglutinin, and gingipains as well, which may directly contribute to its pathogenicity regardless of citrullination (33, 34). It is also important to consider that PD is in fact a polymicrobial disease. \textit{P. nigrescens} is one of the species frequently found in subgingival plaque of aggressive periodontitis mostly at bleeding sites (35, 36). Importantly, elevated levels of \textit{P. nigrescens} DNA fragments have been found in RA serum and synovial fluid as well as in subgingival dental plaque of RA patients with periodontitis (19, 20). Therefore, studying the role of periodontal pathogens other than \textit{P. gingivalis} is crucial for a better understanding of the interaction between the two diseases.

In the experimental settings we used, prolonged PD before CIA was avoided because long-term infection with \textit{P. gingivalis} would potentially cause citrullination of autoantigens and induce ACPA (31), thereby interfering with conclusions regarding relative relevance of T cell modulation. In this context, to our knowledge, our data show for the first time that the presence of concomitant periodontitis aggravates the concurrent T cell–dependent arthritis in vivo. In parallel studies, we found that a single systemic injection of heat-killed \textit{P. gingivalis} and \textit{P. nigrescens} was unable to modulate arthritis, suggesting that aggravation of CIA by concomitant PD could not be assigned to the mere introduction of the bacteria but rather to T cell modulations by the induced PD. Whether repeated exposure to heat-killed periodontal pathogens would be able to aggravate CIA remains to be studies.

Our in vivo experiments revealed a shift in CII-specific Th cells toward Th17, directly correlated with arthritic bone erosion, upon concomitant PD induced by either \textit{P. gingivalis} or \textit{P. nigrescens}. This is in line with Th17 induction by \textit{P. gingivalis} in humans and a previous study showing that intramucosal inoculation of \textit{P. gingivalis} in naive mice can induce a kinin-dependent Th17 phenotype (34, 37, 38). In addition, increased mRNA expression of T cell–related transcription factors has been shown in submandibular lymph nodes of mice upon coinduction of pristane-induced arthritis and \textit{Agregatibacter actinomycetemcomitans}-induced PD (39). Future studies are, however, warranted to clarify whether periodontal bacteria as such may provide an arthritogenic Ag beyond inducing the Th17-polarizing conditions and whether a potential T cell cross-reactivity to a joint-derived Ag and \textit{P. gingivalis}-or \textit{P. nigrescens}-derived epitopes also contributes to the arthritic process.

Irrespective of the Th17 Ag specificity, Th17 cytokines are known to promote arthritis, and the role of IL-17 in osteoclast differentiation and bone erosion is well established (40–42). In addition, Th17 induction at other body sites such as intestinal mucosa has previously been shown to promote experimental arthritis as well (10). Recent studies have reported critical role of IFN-γ/IL-17 double-positive T cells in autoimmune diabetes, characterized by responsiveness to anti–IFN-γ but not anti–IL-17.
Figure 5. *P. gingivalis* and *P. nigrescens* induce a TLR2-dependent Th17 and to a lesser extent Th1 differentiation in vitro, effects that are promoted in the absence of IL-1Ra. (A–F) Splenic CD4+ T cells from wild-type (WT) and IL-1Ra−/− mice were cocultured with irradiated splenic APCs (1:3 ratio) and stimulated with 1 μg/ml Pam3Cys and 10⁷ CFU heat-killed *P. gingivalis* and *P. nigrescens* in the presence of anti-CD3 Abs for 72 h. Th cell differentiation and cytokine profile were measured by flow cytometry (A–C, G, H) and Milliplex cytokine array (D–F). (A) Representative flow cytometry plots showing Th1 and Th17 differentiation in BALB/c WT (upper panel) and IL-1Ra−/− APC + T cell cocultures (lower panel). (B and C) Percentages of IFN-γ– and IL-17–producing CD4+ T cells from WT (B) and IL-1Ra−/− (C) mice. n = 4 per group. (D–F) Cytokine concentrations in the culture supernatants measured by Milliplex. (G and H) CD4+ T cells from IL-1Ra−/− TLR2+/+ and IL-1Ra−/− TLR2−/− mice were cocultured and stimulated as in (A–F). n = 4 mice per group. Statistical differences are based on the Mann–Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001 versus medium or as indicated in (A–F) and compared with WT APC + WT T cell condition in (G, H). n.s., not significant.
FIGURE 6. Schematic illustration of the involvement of concomitant periodontitis in arthritic bone erosion. In addition to the previously suggested induction of ACPA by *P. gingivalis*, both *P. gingivalis* as well as *P. nigrescens* induce Th17 cells, known to promote bone erosion, in an IL-1– and probably IL-6–dependent manner by activating APCs via TLR2. Th1 induction by these pathogens is proposed to be mainly driven via TLR2 expressed on T cells. Furthermore, *P. nigrescens* also diminishes the bone-protective Th2 response.

Treatment of such cells by PD pathogens in vitro, no evidence in this regard was found in our in vivo model. In addition, the clear correlation between IL-17 production and arthritic bone erosion in our study, and the responsiveness of CIA to anti–IL-17 treatment, suggests a greater pathogenic role for IL-17 single-positive (Th17) cells in CIA compared with diabetes models. Nevertheless, in the context of PD–arthritis interaction, it would be interesting to investigate the influence of PD induced by a non-Th17–inducing pathogen to clarify the relative relevance and indispensability of the induced Th17. Also, a Th17–independent effect of periodontal pathogens on bone homeostasis should be considered as well, because a direct TLR2–dependent influence of *P. gingivalis* on osteoclastogenesis has been shown in vitro (45).

IL-1 plays a crucial role in the development of pathogenic Th17 cells, and the absence of endogenous IL-1Ra causes an autoimmune-prone phenotype in mice (46, 47). Our data suggest that both *P. gingivalis* and *P. nigrescens* engage an IL-1–driven Th1 response through stimulation of TLR2 on APCs. This is in line with previous reports describing TLR2 as the main receptor involved in innate response to both bacteria (24, 25). However, with respect to T cell activation, TLR2 is also functionally expressed on T cells themselves, where it plays an important role in proliferation and function of regulatory T cells (8, 48, 49). Our data indicate that, in contrast to Th17 induction, induction of Th1 by periodontal pathogens requires TLR2 directly expressed on T cells. Therefore, presence of distinct cell populations expressing TLR2 at the site of interaction may affect the ultimate T cell profile. The direct activation of TLR2 on T cells by periodontal pathogens to induce Th1 is an interesting finding, as the main effect of TLRs on T cell balance has been attributed to TLR activation on APCs to date (50). In addition, the apparent increase of Th17 cells in conditions where only T cells lack TLR2 (Fig. 5G) may be explained by defective regulatory T cell induction under this condition (48, 49).

It is of interest that, despite similar Th17 induction and joint pathogenicity, *P. gingivalis* and *P. nigrescens* exhibited differential effects on the Th2 response. In contrast to *P. gingivalis*, *P. nigrescens* appeared to be a strong suppressor of the Th2-related IL-4, IL-5, and IL-9 production in vivo. Because *P. nigrescens* was also a weak inducer of IL-10 in DCs and other type 2 cytokines were not produced by these cells, the divergent effects of the two pathogens on type 2 cytokines seem not to be mediated via induction of regulatory cytokines in APCs, but are more likely to be a direct effect on T cells. IL-4 has been shown to inhibit formation of osteoclast-like cells in vivo and to protect from bone erosion during CIA (51, 52). Suppression of IL-4 production upon TLR2 triggering is in agreement with a previous study in which injection of the TLR2 agonist zymosan into arthritis-developing SKG mice was found to suppress IL-4 production by splenocytes (53). Therefore, the net effect of *P. nigrescens* in the context of T cell–driven arthritis seems to rely on a combination of Th2 down-modulation and favoring Th17 differentiation.

Citrullination of proteins by *P. gingivalis* and the subsequent generation of potential autoantigens that drive autoimmunity in RA have been suggested as another possible link between RA and PD (31). A recent study of the complex subgingival microbiota of patients with recent-onset RA, however, found that the presence of *P. gingivalis* in RA is merely associated with the severity of PD but not with ACPA titers (14). The T cell modulation and aggravation of arthritic bone pathology by periodontal bacteria in our study appear to be independent of the possession of citrullinating enzymes or induction of ACPA, because these effects were shared by both periodontal bacteria, whereas citrullination capability is plausibly unique to *P. gingivalis* (32). Furthermore, Abs against major citrullinated peptide candidates, that is, cyclic citrullinated peptide-2, citrullinated fibrinogen, α-enolase, and vimentin, were not induced in the experimental setting used (data not shown). Nevertheless, our study does not preclude the involvement of ACPA in the pathology of RA in humans, nor does it exclude its potential involvement in case *P. gingivalis*-induced PD precedes CIA in other experimental settings. Therefore, translation to human RA requires additional thorough studies in the future, given disease models in animals and the use of CIA as a single model imply certain limitations.

Taken together, this study reveals induction of collagen-specific Th17 and suppression of Th2 phenotypes as relevant pathologic characteristics of periodontal bacteria in the context of arthritis, with a significant impact on arthritic bone erosion. The data further support the relevance of periodontitis in the pathogenesis of arthritis. Further investigation is required to unravel the exact bacteria–host interaction interfaces that serve to modulate the disease.

Disclosures
The authors have no financial conflicts of interest.

References


