

Figure 3.17. Bone deformation is similar between CFA and Pg-CFA groups.

The most severely affected paw was evaluated by micro-computed tomography for bone area present in digits 2, 3 and 4. Representative figures of cortical and periosteal bone delimitation at day 73 of the timeline (43 days after arthritis induction) for (A) vehicle, (B) Pg group, (C) CFA group, and (D) Pg-CFA group. Orange lines outline the cortical bone and bone marrow space in (A) and (B). Orange lines outline the cortical and periosteal bone in (C) and (D); Quantification of (E) periosteal new bone and (F) cortical bone destruction were discriminated based on the bone resolution of 12 μm^3 . Error bars represent $\pm\text{SEM}$ of 7-8 mice/group (ANOVA, $p < 0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

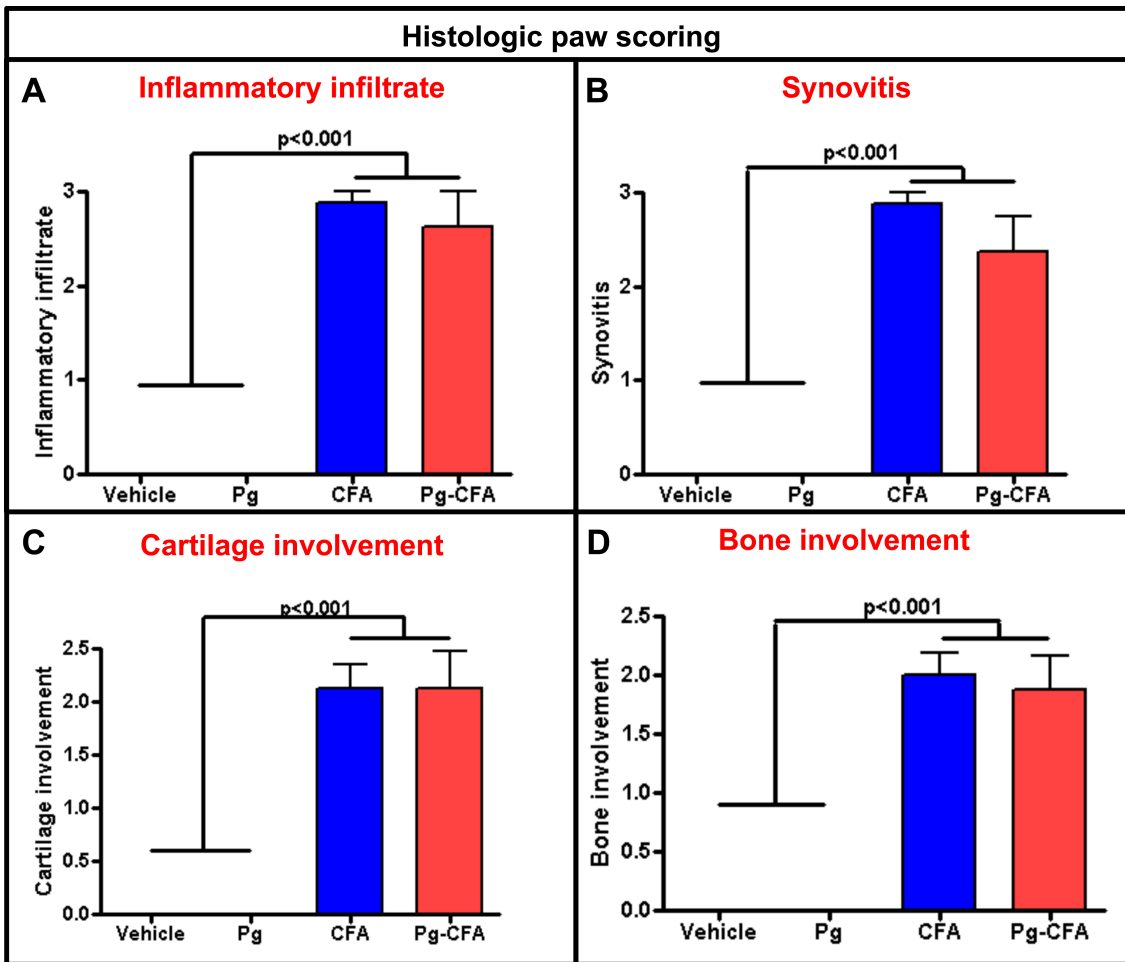


Figure 3.18. Paws showed similar amounts of destruction between mice in the complete Freund's adjuvant group and mice in the Pg-CFA group. Histological sections of the most severely affected paw were blindly scored for (A) inflammatory infiltrate (score 0-3), (B) synovitis (score 0-3), (C) cartilage involvement (score 0-3), and (D) bone involvement (score 0-3). Error bars represent \pm SEM of 8 mice/group (ANOVA, $p < 0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

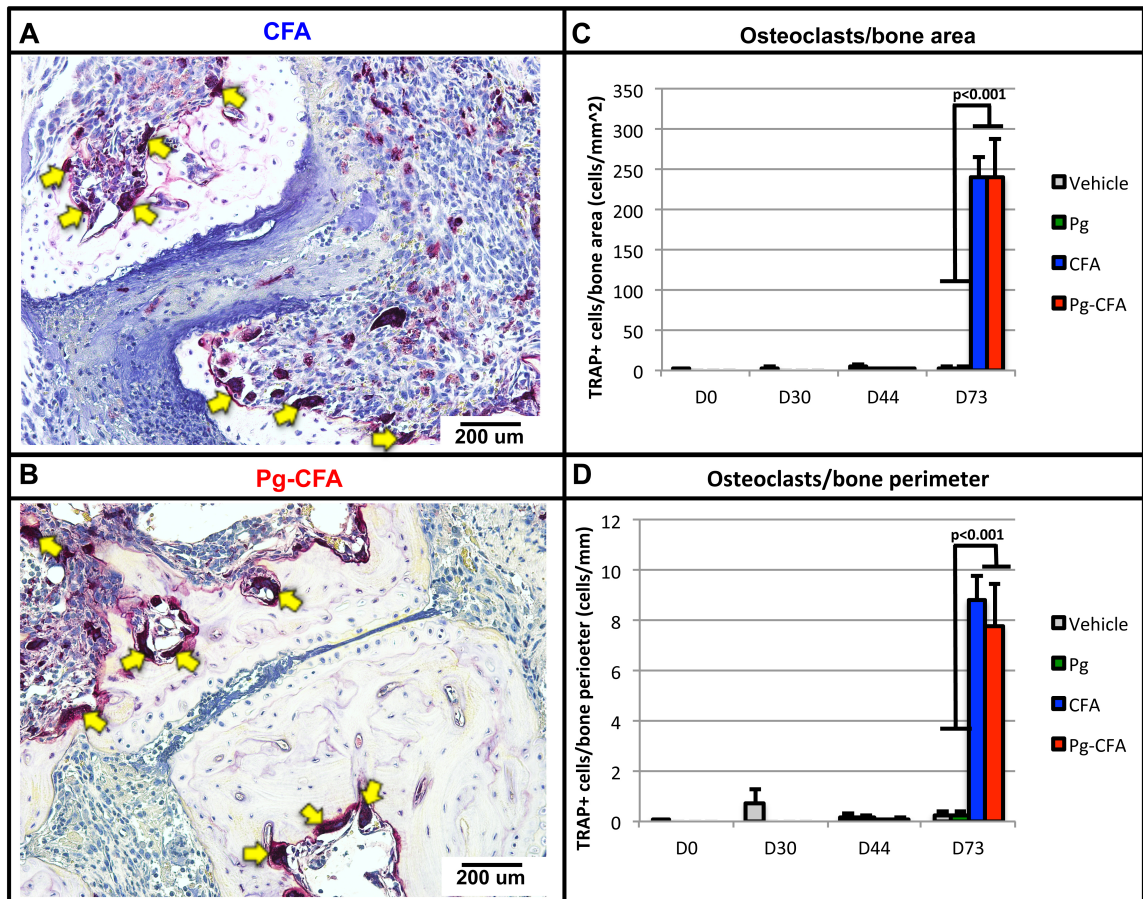


Figure 3.19. Increased osteoclast numbers in arthritic paws in mice immunized with complete Freund's adjuvant (CFA) compared to non-arthritic groups at day 73. Representative figures of joint sections derived from (A) mice induced for arthritis with complete Freund's adjuvant (IFA) and (B) mice that received oral gavages of *P. gingivalis* prior to CFA immunization. Digits 2, 3, and 4 were evaluated for osteoclast number by TRAP-positive staining of cells and represented according to (C) bone area and (D) bone perimeter. Error bars represent \pm SEM of 6-8 mice/group (ANOVA, $p < 0.05$ followed by Tukey's multiple comparison test, indicated in the figures). Group legend: Grey:Vehicle, Green:Pg, Blue:CFA, Red:Pg-CFA.

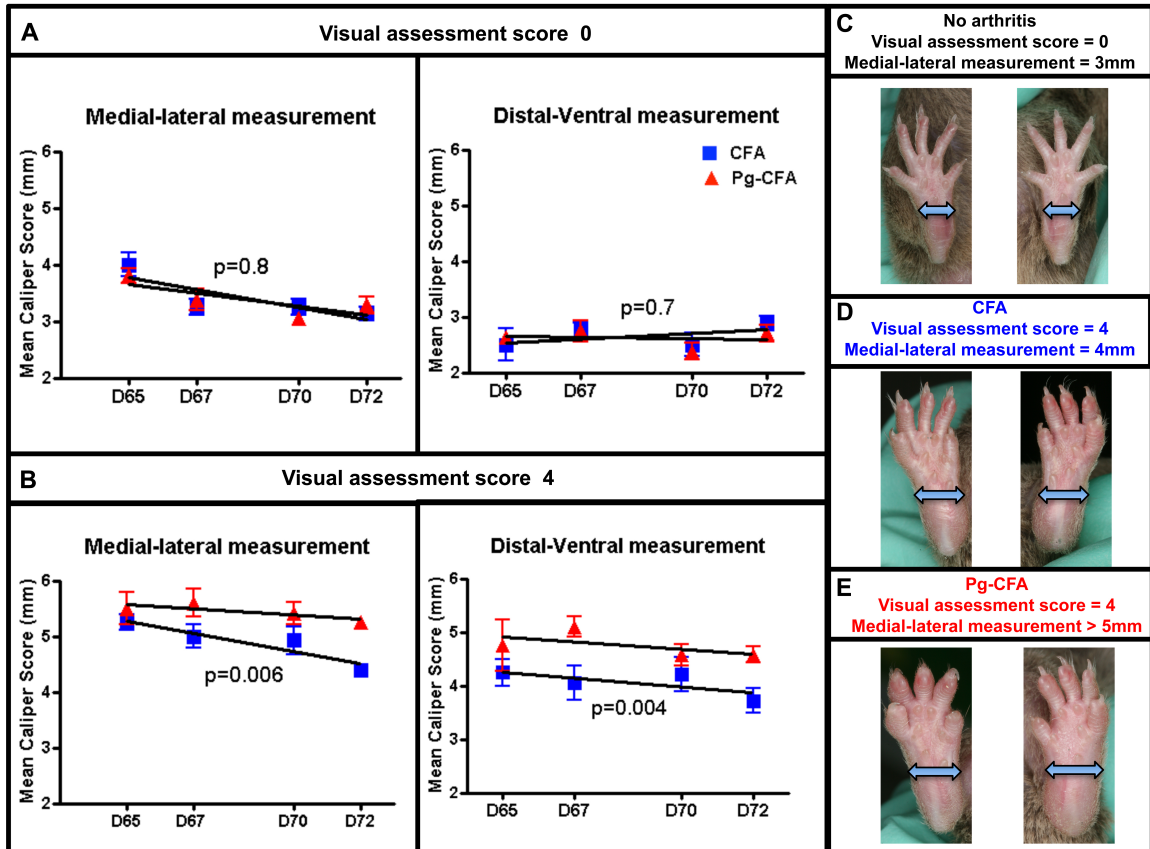


Figure 3.20. Arthritic swelling was higher in mice exposed to *P. gingivalis* prior to CFA immunization. Paws blindly measured with a caliper in the medial-lateral and dorsal-ventral dimensions at timeline days 65, 67, 70, and 72 (35, 37, 40, and 42 after arthritis induction) were compared to the visual assessment scores of (A) 0 (no arthritis) and (B) 4 (arthritis in the entire paw). Representative figures of hind paws that developed (C) score 0 (no arthritis), (D) score 4 with medial-lateral measurement of 4mm in mice from the CFA group, and (E) score 4 with medial-lateral measurements of 5.0mm and 5.5mm in mice from the Pg-CFA group. Error bars represent \pm SEM of 4-6 mice/group (Linear regression analysis, $p < 0.05$).

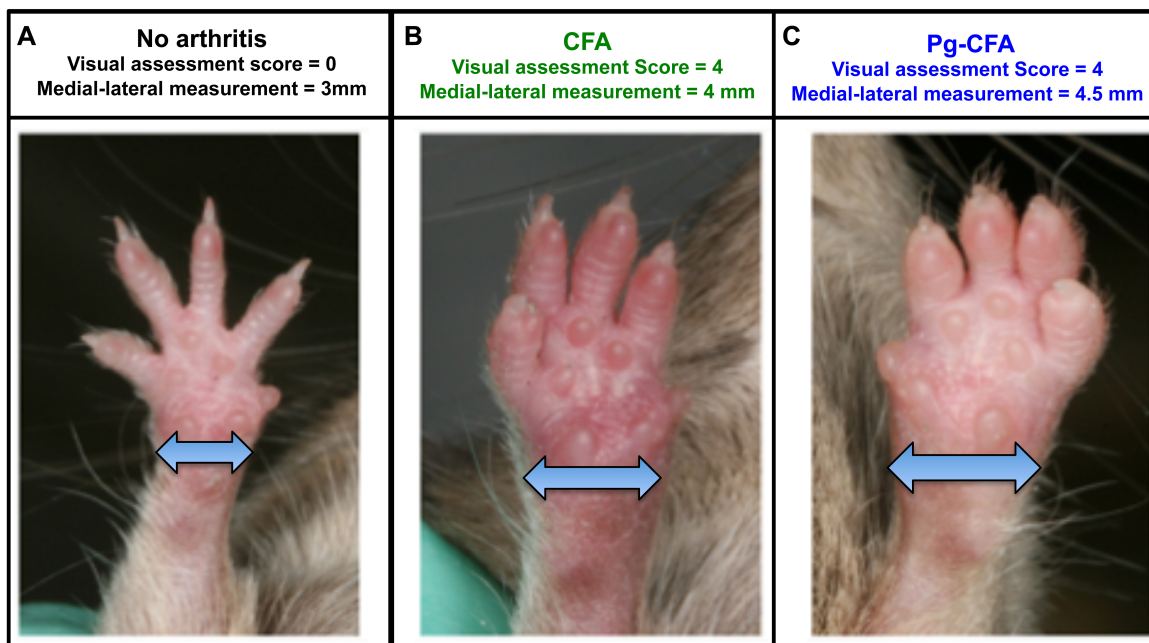


Figure 3.21. Representative figures of front paws that developed (A) score 0 (no arthritis), (B) score 4 with medial-lateral measurement of 4mm in mouse from the CFA group, and (C) score 4 with medial-lateral measurements of 4.5 in mouse from the Pg-CFA group at day 73.

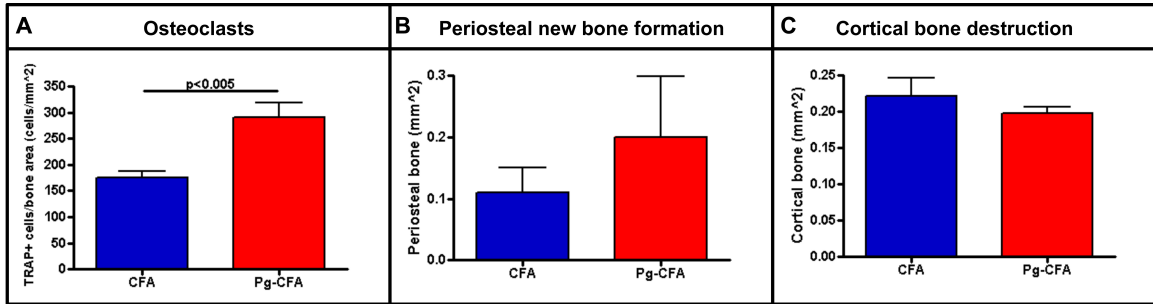


Figure 3.22. Increased osteoclast numbers in mice infected with *P. gingivalis* prior to arthritis induction. Mice induced for arthritis with Complete Freund's adjuvant (CFA) and mice that received oral gavages of *P. gingivalis* prior to CFA were evaluated for (A) osteoclast number by TRAP-positive staining, and (B) periosteal new bone and (C) cortical bone destruction by micro-computed tomography analysis of bone area. Error bars represent \pm SEM of 4-6 mice/group (Student's *t*-test, $p < 0.05$).

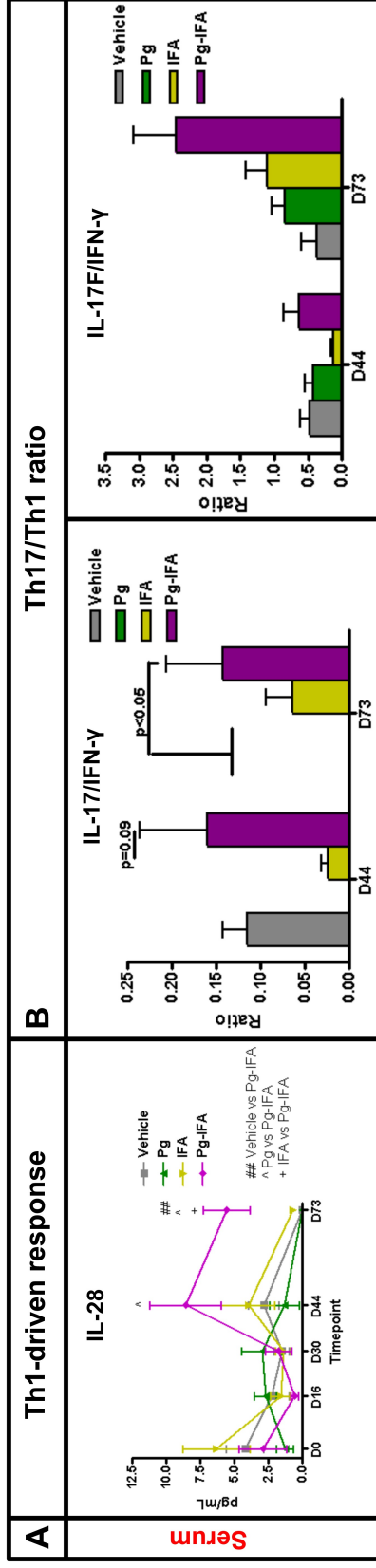


Figure 3.23. IL-28 was up-regulated in mice gavaged with *P. gingivalis* followed by IFA immunization. Murine serum from mice were evaluated by protein array for expression at baseline, D16, D30, D44, and D73 of the timeline for (A) IL-28, IL-17, and IFN- γ . Balance between Th17/Th1 evaluated by (B) IL-17/IFN- γ and (C) IL-17/IFN- γ . Error bars represent \pm SEM of 8 mice/group (ANOVA, $p < 0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

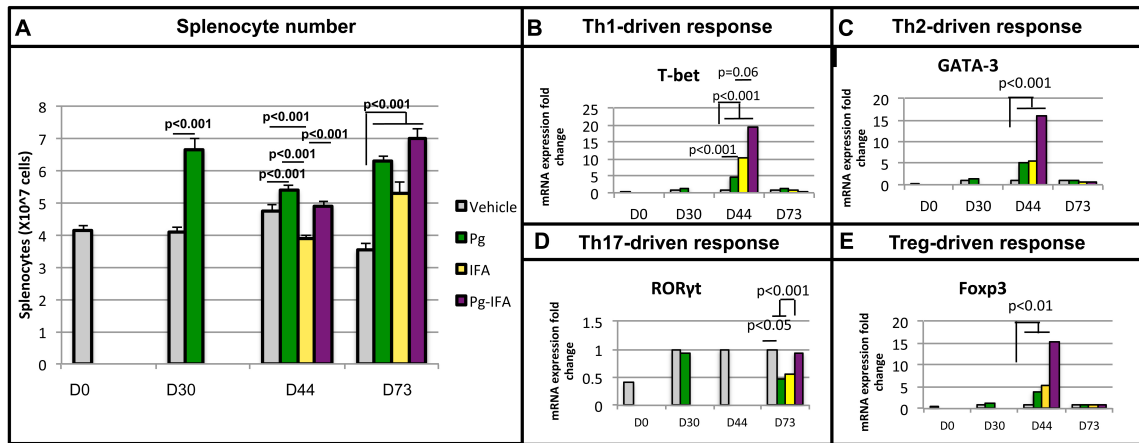


Figure 3.24. Increased systemic immune response induced by *P. gingivalis* and collagen-induced arthritis induced with incomplete Freund's adjuvant. Splenocytes were evaluated for number and transcription factor expression for Th cell development by qRT-PCR at days 0, 16, 44, and 73 of the timeline. Splenocyte numbers represent 8 mice/group, error bars represent \pm SEM (ANOVA, $p < 0.05$). Relative mRNA levels were normalized for housekeeping gene GAPDH. Data is presented as expression fold-change ($n=8$, 2-tailed Student's *t*-test). Group legend: Grey:Vehicle, Green:Pg, Yellow:IFA, Purple:Pg-IFA.

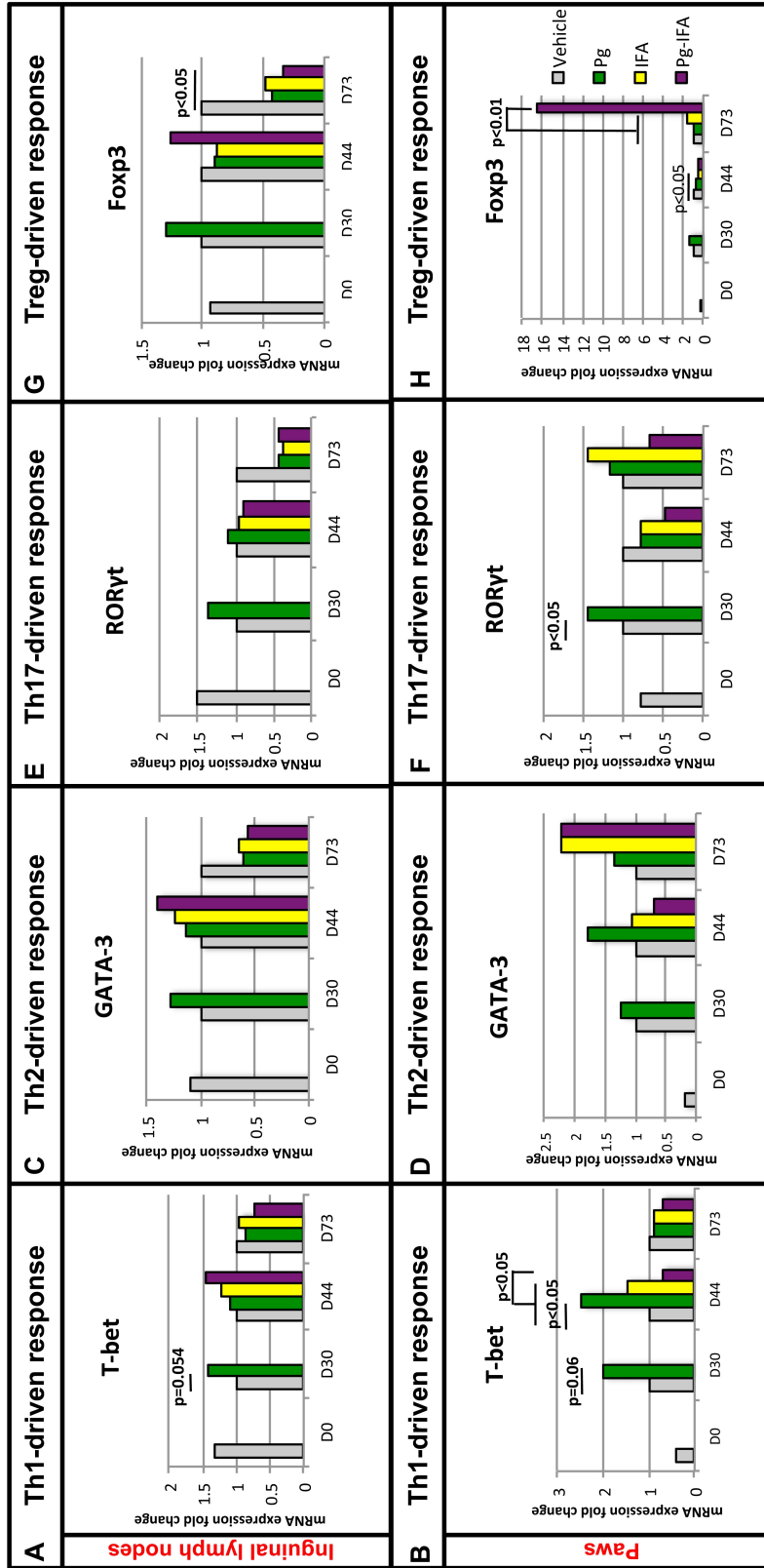


Figure 3.25. Paw Treg response is increased by *P. gingivalis* oral infection in arthritis development with incomplete Freund's adjuvant.

Transcription factors for Th cell development were measured by qRT-PCR for Th1-driven responses by (A-B) T-bet, for Th2-driven responses by (C-D) GATA-3, for Th17-driven responses by (E-F) RORyt, for Treg-driven cell responses by (G-H) Foxp3 at days 0, 30, 44, and 73 of the timeline in the inguinal lymph nodes (upper row) and paws (lower row). Relative mRNA levels were normalized for housekeeping gene GAPDH. Data is presented as expression fold-change (n=8, Student's t-test, p<0.05). Group legend: Grey:Vehicle, Green:Pg, Yellow:IFA, Purple:Pg-IFA.

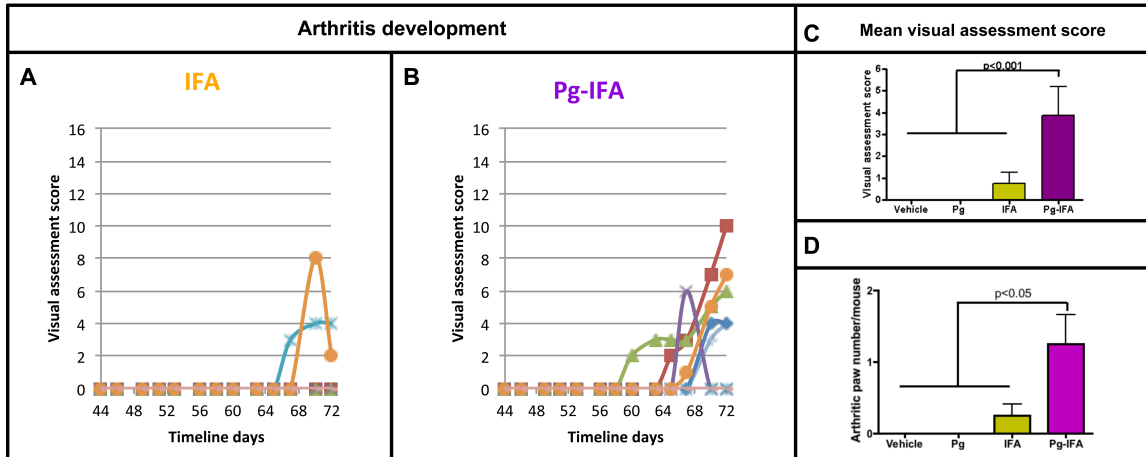


Figure 3.27. Oral *P. gingivalis* increased arthritis progression in the incomplete Freund's adjuvant model for arthritis induction. Arthritis development in (A) mice induced for arthritis with incomplete Freund's adjuvant (IFA) and (B) gavaged with *P. gingivalis* followed by IFA immunization evaluated by the visual assessment score (0-4) at 3 times/week starting from timeline day 44 (14 days after arthritis induction) to day 72 (42 days after arthritis immunization). Each line represents the progression of one mouse, as the sum of the visual assessment score/day. (C) Mean visual assessment score at day 72 (42 days after arthritis induction), and (D) Mean number of paws that developed arthritis by the visual assessment score at day 72 in vehicle, Pg, IFA, and Pg-IFA groups. Data represents \pm SEM of 8 mice/group (ANOVA, $p < 0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

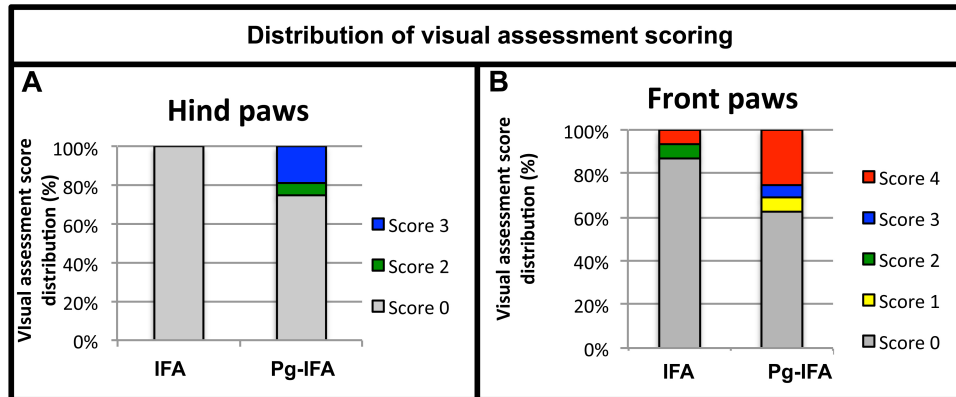


Figure 3.28. Arthritic swelling paw distribution was higher in Pg-IFA compared to IFA alone. Final swelling distribution at 72d of the timeline, or 42 days after arthritis induction, evaluated by visual assessment score in (A) front and (B) hind paws and represented as percentage of each score/group. Data represents 16 front or hind paws/group (Student's *t*-test, $p < 0.05$).

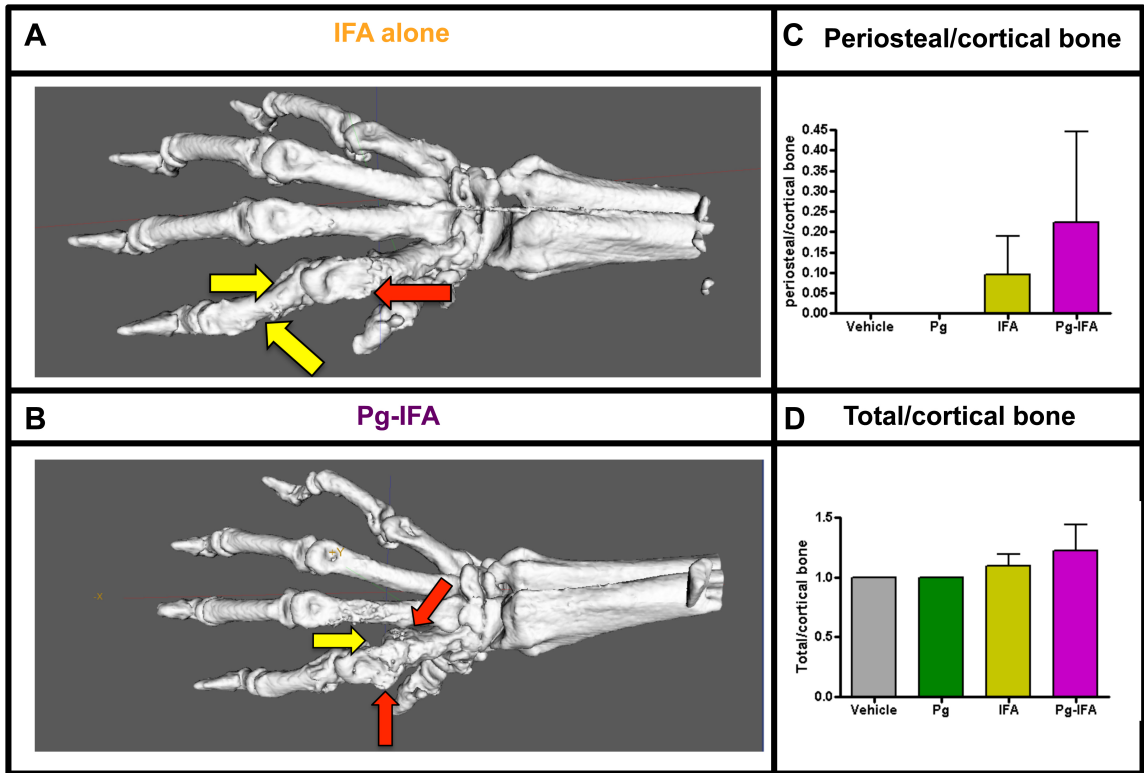


Figure 3.29. Bone deformation is higher in mice gavaged with *P. gingivalis* prior to incomplete Freund's adjuvant immunizations at day 73. The most severely affected paw was evaluated by micro-computed tomography for bone area present in digits 2, 3 and 4. Representative figures of the entire front paw of mice that developed arthritis at day 73 of the timeline (43 days after arthritis induction) of (A) IFA group and (B) Pg-IFA group. Red arrows indicate presence of periosteal new bone and yellow arrows indicate presence of bone resorption. Quantification of (C) periosteal new bone/total bone and (D) total bone/cortical. Periosteal new bone and cortical bone were discriminated based on the bone resolution of $12\mu\text{m}^3$. Error bars represent $\pm\text{SEM}$ of 7-8 mice/group (ANOVA, $p < 0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

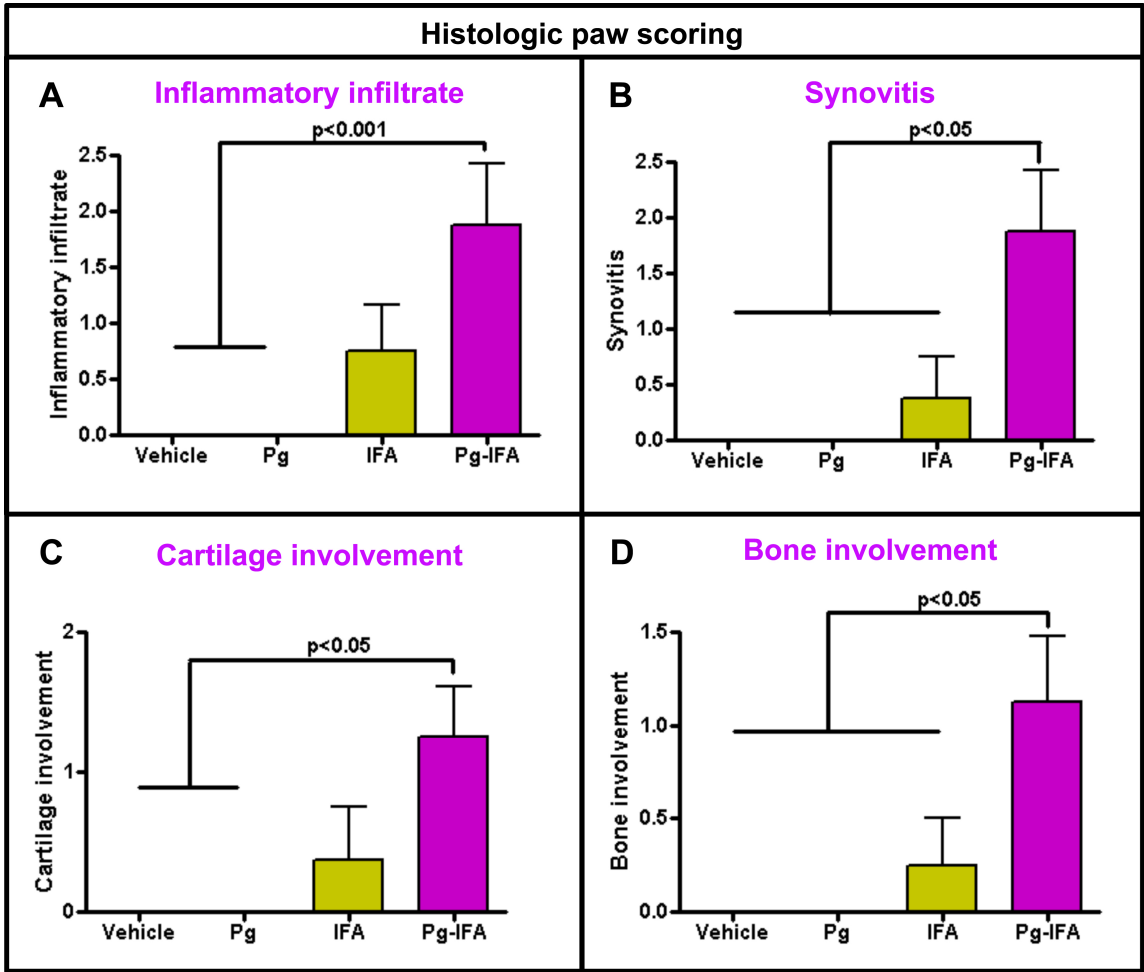


Figure 3.30. Paws showed higher destruction in mice orally infected with *P. gingivalis* followed by immunization with collagen II and incomplete Freund's adjuvant (IFA). Histological sections of the most severely affected paw were blindly scored for (A) inflammatory infiltrate (score 0-3), (B) synovitis (score 0-3), (C) cartilage involvement (score 0-3), and (D) bone involvement (score 0-3). Error bars represent \pm SEM of 8 mice/group (ANOVA, $p < 0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

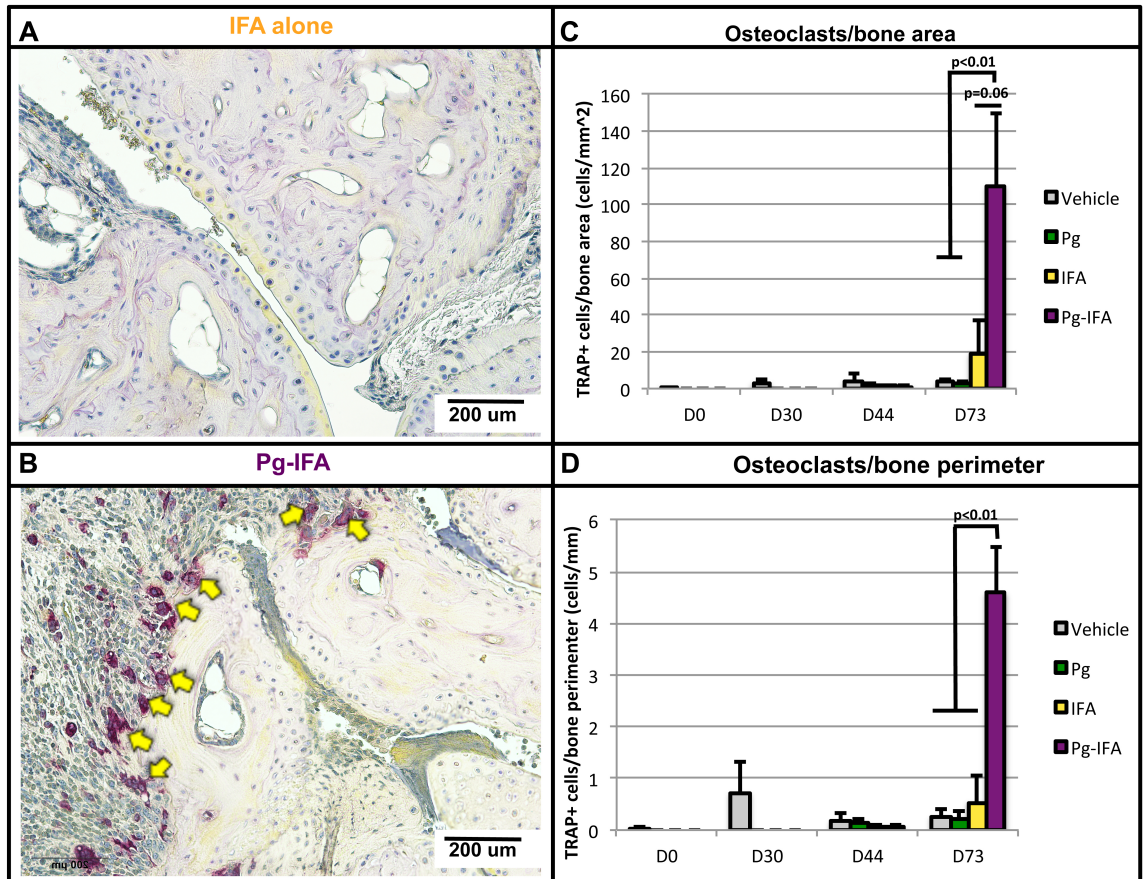


Figure 3.31. Increased osteoclast numbers in mice infected with *P. gingivalis* prior to arthritis induction with incomplete Freund's adjuvant at day 73. Representative figures of joint sections derived from (A) mice induced for arthritis with incomplete Freund's adjuvant (IFA) and (B) mice that received oral gavages of *P. gingivalis* prior to IFA immunization. Digits 2, 3, and 4 were evaluated for osteoclast number by TRAP-positive staining of cells and represented according to (C) bone area and (D) bone perimeter. Error bars represent \pm SEM of 8 mice/group (ANOVA, $p < 0.05$ followed by Tukey's multiple comparison test, indicated in the figures). Group legend: Grey:Vehicle, Green:Pg, Yellow:IFA, Purple:Pg-IFA.

	Day 0	Day 16	Day 23	Day 30	Day 37	Day 44	Day 51	Day 58	Day 65	Day 73
Baseline	0/8									
D30 Vehicle	0/8			0/8						
D30 Pg	0/8	6/8	6/8	4/8						
D44 Vehicle	0/8			0/8						
D44 Pg	0/8	8/8	8/8	8/8	8/8	8/8				
D44 CFA	0/8					0/8				
D44 Pg-CFA	0/8	8/8	8/8	8/8	8/8	8/8				
D44 IFA	0/8					0/8				
D44 Pg-IFA	0/8	8/8	8/8	8/8	8/8	6/8				
D73 Vehicle	0/8									0/8
D73 Pg	0/8	8/8	8/8	8/8	7/8	8/8	8/8	8/8	6/8	8/8
D73 CFA	0/8									0/8
D73 Pg-CFA	0/8	6/8	7/8	8/8	8/8	8/8	6/8	8/8	8/8	0/8
D73 IFA	0/8									0/8
D73 Pg-IFA	0/8	4/8	8/8	8/8	5/8	8/8	7/8	2/8	6/8	0/8

Table 3.1. *P. gingivalis* oral colonization during periodontitis and arthritis development. Murine oral microflora collected at timeline days 0, 16, 23, 30, 37, 44, 51, 58, 65, and 73 were evaluated by PCR of Arg-gingipain (201 bp). Mice from all groups were evaluated. Values represent the number of infected mice/number of total mice in each group (n=8). Blue rows represent days in which the analysis was performed and grey rows represent timepoints that were not evaluated.

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CHAPTER IV

SYSTEMIC CIRCULATORY MICROBIAL COMPONENTS AND IMMUNE REGULATORS IN PATIENTS WITH PERIODONTAL DISEASE AND RHEUMATOID ARTHRITIS – WORK IN PROGRESS

INTRODUCTION

Microorganisms that invade the host are first detected by a family of pathogen-recognition receptors that detect conserved microbial components called pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006). These PAMPs represent molecules vital for microbial survival such as flagellin, nucleic acid structures unique to bacteria and viruses (CpG DNA, dsRNA), and the bacterial cell wall LPS, lipoteichoic acid, and peptidoglycan. The detection of PAMPs by pathogen-recognition receptors (PRRs) will activate multiple pro-inflammatory signaling pathways to mount an effective antiviral or bactericidal response targeting the invading microbes. However, in periodontal disease the invading microorganisms are not removed because of the complex plaque/biofilm formation leading to a continuous source of activators of the innate immune response and chronic expression of inflammatory cytokines into the bloodstream.

Several studies have confirmed this by demonstrating a higher expression of toll-like receptors (TLRs) in periodontitis compared to control, in addition to its synergism with nod-like receptors (NLRs). Currently, the only methods available to detect microbial components in human samples are the limulus amoebocyte lysate assay for bacterial LPS, DNA analysis, or direct bacterial cultivation (Forner et al., 2006; Moen et al., 2006). While direct microbial cultivation does not allow a complete analysis of the oral microbiota, some studies have identified bacterial LPS and DNA in the blood of patients with severe periodontal disease. However, a variety of other microorganism components may be present in the bloodstream of these patients, which would lead to activation of the innate immune response in other sites of the body. Therefore, it is important to clarify the microorganism composition of the blood of patients with periodontal disease and how this may potentially affect the immune system via immune regulators. In this proposal, we will utilize cells with the presence or absence of pathogen-associated patterns as tools for identifying the presence and biological significance of microorganism components in the blood of patients with periodontal disease and rheumatoid arthritis.

GENERAL HYPOTHESIS

Patients with severe periodontal disease have an increased potential of activating Th17 cell-driven responses in arthritic patients via microorganism components in the plasma compared to healthy patients.

To address this question, we propose the following specific aims:

Specific Aim 1: Identify the immune responses detectable in plasma from periodontitis patients that may influence arthritis development.

Specific Aim 2: Characterize the pro-inflammatory activity of microorganism components in the plasma of patients with periodontitis.

MATERIALS AND METHODS

Subject Population

A total of 100 adult subjects will be recruited. These subjects will be comprised of four patient populations (n = 20/group, except for healthy population which will have 40): 1) healthy population; 2) severe periodontal disease population; 3) rheumatoid arthritic population; 4) combined severe periodontal disease and rheumatoid arthritic population.

Periodontal Disease

Healthy and periodontitis will be classified as previously described by our group (Ramseier et al., 2009). The following clinical parameters will be measured at 6 sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual) for up to 168 sites in each subject: 1. Presence of plaque recorded dichotomously as 0 (absence) or 1 (presence); 2. Degree of gingival redness

recorded dichotomously as 0 or 1; 3. PD measured from the gingival margin to the base of the pocket in mm; 4. Clinical attachment level measured from the cementum-enamel junction to the base of the pocket in mm; 5. BOP recorded dichotomously as 0 or 1; and 6. Suppuration recorded dichotomously as 0 or 1. If the patient clinically qualifies for the study, a full-mouth series of digital radiographs will be taken (14 periapical radiographs and 4 bitewings), according to periodontal standard care, unless the patient can provide comparable radiographs taken within three years of the start of the study.

Rheumatoid Arthritis

Patients will be diagnosed for rheumatoid arthritis by a rheumatologist, in accordance with the American College of Rheumatology revised criteria as follows: presence of synovitis in at least 1 joint, absence of an alternative diagnosis that better explains the synovitis, and achievement of a total score of 6 or greater (of possible 10) from the individual scores in 4 domains: number and site of involved joints (score range 0-5), serologic abnormality (score range 0-3), elevated acute-phase response (score range 0-1), and symptom duration (2 levels; range 0-1) (Aletaha et al., 2010). This new classification system redefines the current paradigm of RA by focusing on features at earlier stages of disease that are associated with persistent and/or erosive disease, rather than defining the disease by late-stage features.

Data collection

Periodontal disease measurements, biofilm samples, and arthritis measurements will be collected before prophylaxis is performed (Fig. 4.1). A biofilm sample of the deepest site of the most affected tooth in each sextant will be collected from patients as described (Beikler et al., 2006). A total of forty mL of blood will be collected into EDTA coated tubes, with 20 mL of collected before and 20 mL collected after prophylaxis. The second blood collection will be performed within 5 min after a 30-45 minute oral prophylaxis, according to Fig.4.1. The 5 min period is based on previous studies showing that the total number of bacteria and LPS present in the bloodstream is higher shortly after oral manipulation (Kinane et al., 2005). The plasma samples will then be placed in 1 mL tubes, and stored at -80°C. Plasma will be isolated instead of serum given that the rate of microorganism recovery from plasma cultures is significantly greater than from serum cultures (Wormser et al., 2000).

Data analysis

Specific Aim 1: Identify the immune responses of plasma from periodontitis patients that may influence arthritis development. Peripheral blood mononuclear cells (PBMC) will be isolated from healthy patients by the Ficoll-Hypaque method as described (Fuss et al., 2009). PBMC will be plated at 2.5×10^5 cells/well with 25 μ L RPMI-10 complete media and stimulated with 25 μ L of plasma patient samples for 3h and 12h. For the 3h stimulation, a 36K gene microarray (Affymetrix Human Genome U219 Array Strip) will provide a readout for potential activators and inhibitors of Th17 cells. Protein analysis by ELISA

after 12h of PBMC stimulation with plasma samples will confirm the gene expression finding. A total of 6 patients/group will be evaluated for the gene microarray, and all samples will be evaluated for the protein array.

After identification of the immunoregulator(s), purification will be performed by gel filtration and ionic exchange chromatography in combination with high performance liquid chromatography (HPLC) at the Core Facility of the University of Michigan. Electrospray ionization mass spectrometry (ESI-MS) will be used for final purification and identification of the immuno-regulator(s) at the Core Facility of the University of Michigan. The inflammatory markers of RA will be compared to the immunological and inflammatory profile found in the in-vitro setting.

Specific Aim 2: Characterize the pro-inflammatory activity of microorganism components in the plasma of patients with periodontitis. A bioassay system will be established using human embryonic kidney (HEK) 293T cells stably transfected for 24h with expression plasmids as described previously (Hasegawa et al., 2006). This human cell line was chosen because it is easily transfectable and does not express PRRs. The cells will be transfected with the following PRRs: TLR2/1, TLR2/6, TLR5, TLR4/MD2, TLR9, Nod1, Nod2, and a control empty vector. Cells will be incubated with 25uL of Dulbecco's modified Eagle media supplemented with 10% of certified fetal bovine serum, 1% of streptomycin and 1% penicillin along with 25uL of patient-derived plasma for 12h. Highly purified fractions of whole soluble Cellosyl-treated peptidoglycan, synthetic compounds γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP),

muramyl-dipeptide (MDP), sBLP (Pam₃-Cys-OH), CpG, and poly(I:C), and LPS free of Nod-ligands will be used as control ligands. Ligand-dependent NF-κB activation will be determined using HEK293T cells transfected with expression plasmids in the presence of firefly luciferase as a reporter and control beta-galactosidase. For determination of the pro-inflammatory competence, lysates will be evaluated for the expression of TNF-α and CCL2 (MCP-1) by sandwich ELISA assays. Transfected cells that express receptor activation will indicate that a microbial component that binds to the specific receptor is present within the patient plasma. In addition, macrophages derived from the bone-marrow of 6-week-old B6 wild-type mice and mice lacking specific PRRs will also be utilized. The cells will be derived from mice: TLR2^{-/-}, TLR3^{-/-}, TLR5^{-/-}, TLR6^{-/-}, TLR9^{-/-}, Nod1^{-/-}, Nod2^{-/-}, and double knockout MyD88^{-/-} /TRIF^{-/-}. Cells will be incubated with 25uL of RPMI 1640 media supplemented with 10% of certified fetal bovine serum, 1% of streptomycin and 1% penicillin along with 25uL of patient-derived plasma for 12 h. Receptor activation will be identified by expression of TNF-alpha and CXCL2. After classification of the microbial component(s) in the plasma via receptor activation, the microbial component (s) will be purified by column chromatography and identified by time-of-flight mass spectrometry (TOF-MS) at the UM Protein Core Facility.

RESULTS

A total of 40 patients have completed the study. Demographics can be seen in table 4.1.

Forty serum samples without prophylaxis were evaluated by ELISA for the presence of LPS and expression of several cytokines, including IL-1 β , MCP-1, IL-8, IL1-Ra, and TNF- α . Although the levels in both groups were low, there was a statistical difference observed between groups for TNF- α at baseline and 6 months after the study started (Fig. 4.2). Both endothelial cells and PBMC are biologically sensitive cells that express cytokines upon innate immune receptor activation. Still, while endothelial cells did not seem to identify differences between serum samples from patients, PBMC stimulated with serum samples derived from periodontitis patients showed a decreased expression of IL-6 compared to healthy patients (Fig. 4.3). This finding indicated that an inhibitor of IL-6 is potentially present in the serum samples of patients with periodontitis.

CONCLUSION

These results indicate that the biological composition of serum samples from healthy and patients with severe periodontitis is distinct.

This proposed feasibility study will aid in providing the impetus for sample size and other design requirements for larger, more expanded human clinical trial testing. The literature indicates that a variety of microorganism components may be present in the bloodstream of patients with severe periodontitis, leading to activation of the innate immune response in the bloodstream and/or other sites of the body. Since RA and PD show clinical associations, it is important to clarify the microorganism composition of the serum of periodontitis patients and how

this may potentially affect the immune system via immune regulators in arthritic patients.

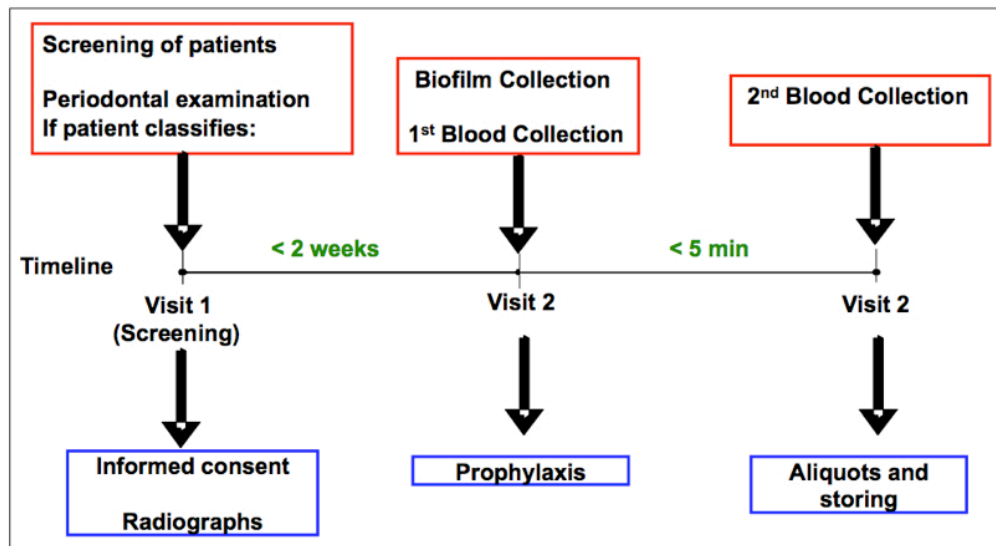


Figure 4.1. Schematic diagram illustrating the study experimental design.

One hundred systemically healthy patients will be divided into 4 groups, with 20 patients per group: 1) Healthy oral cavity, 2) Severe periodontitis, 3) Rheumatoid arthritis, or 4) Combined severe periodontitis and rheumatoid arthritis. Visit 1 will consist of a screening of the patient by disease classification and inclusion/exclusion criteria, followed by a periodontal examination. If the patient qualifies, assignment into one of the four groups described will occur. Patients will sign the informed consent and have radiographs taken for diagnostic purpose. Within a period of 2 weeks, the patient will return for visit 2. Biofilm collection and blood collection will be performed, followed by a 30-45 min prophylaxis procedure. Within 5 min of the end of prophylaxis procedure, a second blood collection will be performed. Blood samples will be centrifuged to obtain plasma, placed into 10 aliquots, and stored at -80°C .

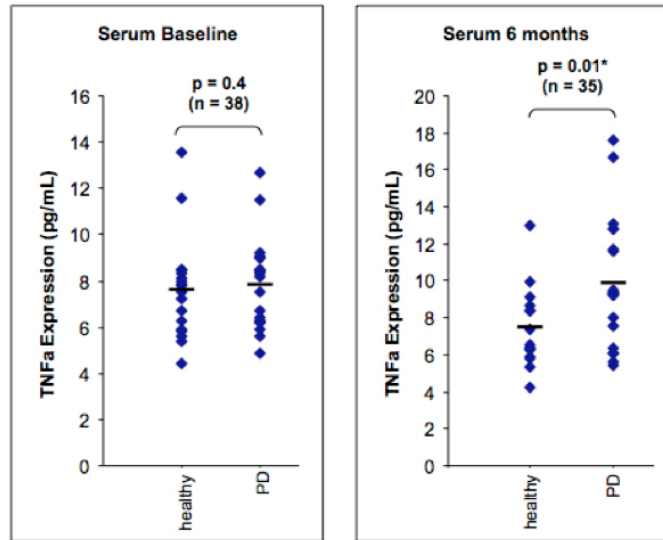


Figure 4.2. Increased TNF- α in serum samples from patients with periodontitis. Serum samples derived from patients with a healthy oral cavity and severe periodontitis were evaluated for the presence of TNF- α . A statistical difference was observed at 6 months after the study initiated, with a higher expression of TNF- α being observed in the severe periodontal disease group. (Student's *t*-test, $p < 0.01$).

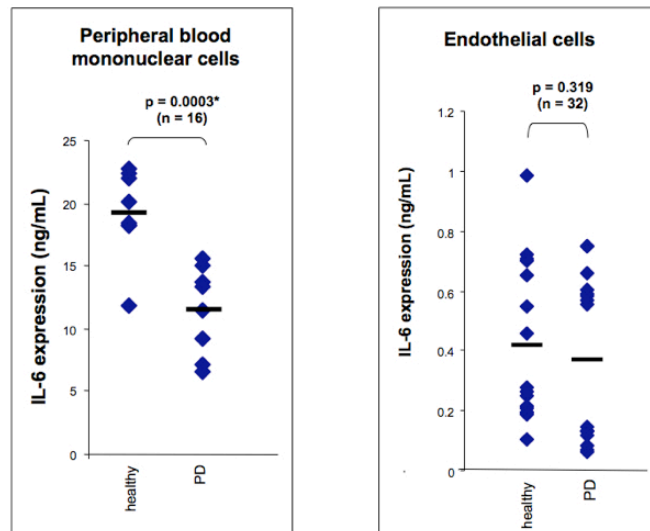


Figure 4.3. Decreased IL-6 expression from peripheral blood mononuclear cells (PBMCs) stimulated with serum from patients with severe periodontitis. Primary human PBMCs derived from a healthy donor and human endothelial cells were stimulated with serum derived from patients with severe periodontal disease and healthy controls. The supernatants derived from the peripheral blood mononuclear cells showed a statistical difference between stimulation with healthy and periodontitis serum. Patients with severe periodontitis had a lower expression of IL-6 compared to healthy controls (n = 16, Student's *t*-test, $p < 0.001$).

	Healthy	Severe Periodontal Disease	p Values
Subjects (n)	20	20	NA
Males (%)	30	50	0.103
Caucasian (%)	95	85	0.152
Current tobacco users (%)	15	25	0.221
Former tobacco users (%)	35	70	< 0.05
Mean number of teeth	26.8	24.45	< 0.05
Mean age (years)	46.1	53	< 0.05
Sites with BOP (%)	10.46	60.08	<0.001
Sites with gingival redness (%)	31.57	83.42	<0.001
Sites with plaque (%)	41.04	81.8	<0.001
Mean PD (mm)	1.83	3.27	<0.001
Sites with PD >4 mm (%)	0	20.42	<0.001
Mean CAL (mm)	0.7	3.45	<0.001

Table 4.1. Demographics of healthy patients and patients with severe periodontal disease.

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CHAPTER V

GENERAL CONCLUSIONS

Studies show that periodontal disease treatment can influence the clinical parameters of RA. The first aim of this dissertation was to determine the oral colonization, alveolar bone loss, and splenic responses in mice of 3 different strains of *P. gingivalis*. We confirmed previous literature reports that the *in vitro* response of *P. gingivalis* was similar among strains, but the systemic effect was distinct. Protection from alveolar bone loss was associated to high splenic anti-inflammatory IL-10 expression. The systemic alteration observed could have been caused either directly via *P. gingivalis* or by other bacteria present in the microflora once *P. gingivalis* colonized the oral cavity (keystone hypothesis). Strain A7A1-28 has a divergence in the genome of 3.5% when compared to the genetically closely related strains W83 and W50 (Igboin et al., 2009). These differences can account for virulence factors that may influence the systemic spreading and activation of other cells. Our results indicated that the IL-10 expression was not attributed to Tregs or Bregs in the spleen, indicating that the source of IL-10 may be the Tr1 subset. Mechanistically, it is possible that the oral infection altered the systemic response by 3 pathways: 1) bacterial products and/or, 2) hyper-activating/priming cells, and/or 3) cytokine/chemokine systemic

expression (Fig. 1.2). Based on these findings and the consistency of oral infection, we further evaluated strain W83 effect in arthritis.

In the second aim, we determined the effect of chronic periodontal disease on immune activation during collagen-induced arthritis (CIA) development in mice. The timing of induction of both diseases resulted in mice with more swollen joints and more bone destruction, which is in accordance to the clinical scenario indicated by several studies (Erciyas et al., 2012; Ortiz et al., 2009). CIA immunizations alone resulted in periodontal bone loss, as confirmed by previous studies (Park et al., 2011), indicating that an alteration of the oral microflora occurs once arthritis is induced. Prior *P. gingivalis* oral infection resulted in a trend for increased serum Th17/IFN- γ ratio and increased splenocyte numbers (splenomegaly) in mice induced for CIA. Our general conclusion is that *P. gingivalis* invaded the epithelium and induced a local immune response in the gingival tissues and submandibular lymph nodes (Fig. 5.1). The infection generated in the oral cavity sensitized the leucocytes in the blood and spleen. When arthritis was induced, the sensitization of the blood's leukocytes changed the Th17/Th1 balance, favoring a Th17-driven response. The subsequent local immune activation that initiated in the inguinal lymph nodes did not seem to be altered by *P. gingivalis* infection. However, once the spleen initiated the immune responses towards collagen II, the sensitized cells increased the Th17 cell response via increased IL-1 β , IL-6, IL-21, IL-22, IL-23, and TGF- β . The increased response seen in mice induced for both periodontal disease and CIA was associated to higher amount of paw swelling and osteoclast numbers. Therefore,

the response that ultimately developed in the paws favored the Th17 cell development. Th17 cells stimulate osteoclasts by inducing RANKL on osteoblasts and synoviocytes via IL-17. RANKL binds to RANK in osteoclasts and leads to osteoclastogenesis and bone resorption (Takayanagi, 2010). Therefore, the mechanism by which periodontal disease influenced arthritis development was via sensitized cells in the serum and spleen of mice that ultimately favored a Th17 cell response.

Still, phenotypic and splenic differences were also observed between mice infected with *P. gingivalis* in combination to either complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA). The reason to utilize both CFA and IFA to evaluate *P. gingivalis* infection effects in arthritis was that the presence of *M. tuberculosis* at the time of immunization assists in inducing a florid response towards collagen II (Billiau and Matthys, 2001). Therefore, we anticipated that the florid response would not allow evaluation of a co-factor influencing arthritis development. However, our results showed that mice immunized with either CFA or IFA had arthritis development influenced by *P. gingivalis* oral infection. The major differences were that the time points affected by *P. gingivalis* infection were earlier in the IFA groups compared to the CFA groups. In the CFA model, *P. gingivalis* infection induced phenotypic differences starting at day 65 (Fig. 3.20) compared to day 57 in the IFA group (Fig. 3.27). An increased splenic cell number was observed at day 73 in CFA (Fig. 3.10) and in both days 44 and 73 in the IFA group (Fig. 3.24). Splenic cytokine expression was mostly increased at day 73 in the CFA group (Fig. 3.12) when combined with *P. gingivalis*, while

these changes were mostly observed at day 44 in the IFA group when combined with *P. gingivalis* (Fig. 3.26). These results show that if *M. tuberculosis* was present at the time of immunization, the effects of *P. gingivalis* were blunted at earlier timepoints (day 44). Still, the effect of sensitized cells could be observed once the arthritic response started diminishing over time (days 65 and 73). Once *M. tuberculosis* was removed from the immunization process, the sensitization caused by *P. gingivalis* could be seen earlier in the splenic cytokine response (day 44) and paw arthritic development (day 57). These results show that the factors involved in immunological initiation of arthritis influence the effect of periodontal disease in arthritis progression. Still, *P. gingivalis* oral infection could influence CIA by immunizations with either CFA or IFA. In the clinical scenario, this means that *P. gingivalis* chronic oral infection could sensitize immune cells and influence arthritis development if breach of tolerance occurs by a strong or light component. These results are important in furthering our understanding for the potential of an oral chronic infection in altering arthritis condition in susceptible patients, and may have important implications for developing future preventive periodontal therapies.

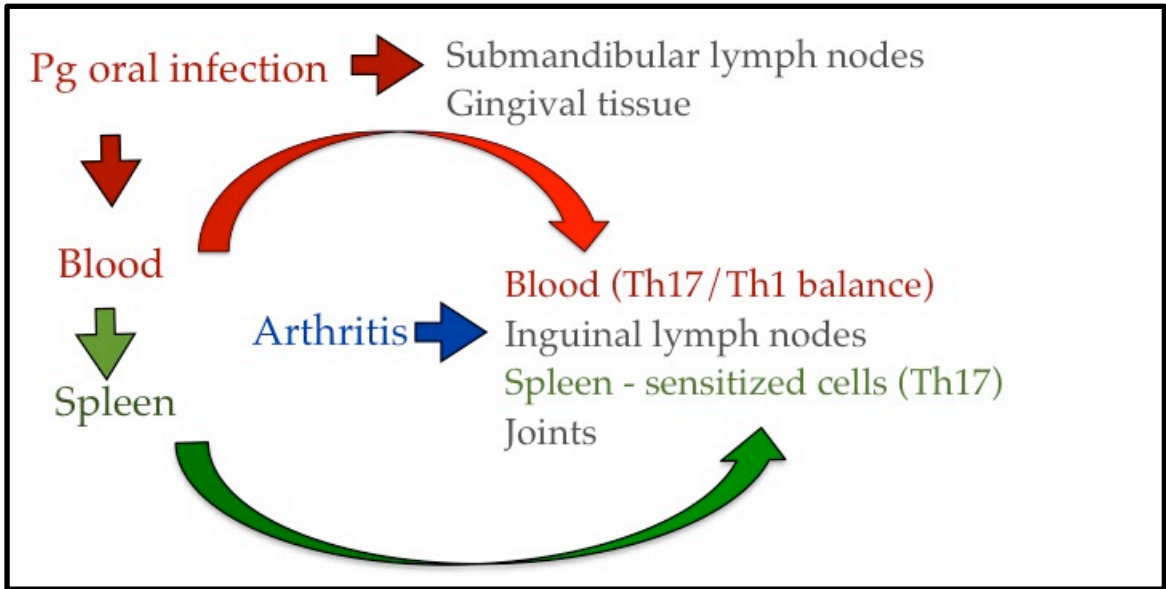


Figure 5.1. Schematic figure of the influence of murine periodontal disease in collagen-induced arthritis development. *Porphyromonas gingivalis* (*Pg*) oral infection lead to chronic oral inflammation by activating immune cells in the submandibular lymph nodes and gingival tissue. The oral infection also activated and sensitized immune cells in the blood and spleen. Arthritis induction initiated in an altered serum cytokine environment favoring Th17 cell development. The response towards collagen II initiated in the inguinal lymph nodes that did not seem affected by the oral infection. The development of the arthritic immune response then initiated in a sensitized environment in the spleen, also favoring a Th17 response, which ultimately affected the immune response generated in the joints.

FUTURE DIRECTIONS

Our results indicated that *P. gingivalis* strain A7A1-28 had the lowest amount of bone loss, which was associated to high levels of splenic IL-10. Since IL-10 has an anti-inflammatory effect, it would be interesting to explore how this particular *P. gingivalis* strain may affect arthritis development in mice. Results from this dissertation combined to the literature suggest that the timing between periodontitis and arthritis induction could be an important determinant in evaluating the interaction between both diseases. When infected prior to arthritis development, *P. gingivalis* worsened the arthritis condition (Cantley et al., 2011). However, when infected after arthritis induction, no differences in arthritis development were observed (Trombone et al., 2010). Therefore, the timing between the initiations of both diseases may have influence in the final clinical outcome.

Mice induced for arthritis alone showed alveolar bone loss and Th cells activation of submandibular lymph nodes. Further analysis on the microbial composition of mice induced for arthritis is of interest for evaluating the effect that arthritis may have in periodontal disease development.

While *P. gingivalis* seems to have important implications in the clinical scenario for RA, evaluation of other periodontal bacteria in arthritis development need further investigation, including multi-infections.

P. gingivalis infection induced cytokine changes mainly by the Th17 polarization during arthritis development. Therefore, administration of antibodies

against IL-17 and up-stream cytokines in mice with combined diseases may result in interesting findings that have important clinical relevance.

In addition, human studies that assist in defining how periodontal disease may influence arthritis development are of interest. To assist in defining these mechanisms, we proposed evaluating patients with RA and periodontal disease for microorganisms and their components present in their bloodstream, and the biological effect that these may have in the immune response.

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