ORAL *PORPHYROMONAS GINGIVALIS* INFECTION INCREASES ARTHRITIS SEVERITY AND PROGRESSION

by

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DEDICATION

To my husband, son, mother, father, and sister -

For continuously supporting my academic endeavors through the years.

I owe it all to you.

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ABSTRACT

Periodontitis is a polymicrobial oral infection characterized by the destruction of tooth supporting structures that may influence rheumatoid arthritis. *Porphyromonas gingivalis (P. gingivalis)*, a bacterium implicated in the etiology of periodontitis, has shown variation in inducing T-cell responses depending on the clinical strain. In this study, we determined the differences between the systemic responses of different *P. gingivalis* strains that have important clinical representation. We also determined the effect of chronic periodontal disease on immune activation during collagen-induced arthritis (CIA) development in mice. Our results confirmed that the strains of *P. gingivalis* A7A1-28, W50, and W83 had the ability to colonize the oral cavity of mice, induce periodontal disease, and change the expression profile of splenocytes. We found that splenic anti-inflammatory IL-10 expression was associated with the least amount of alveolar bone loss. Our results showed that *P. gingivalis* had the ability to activate dendritic cells and express IL-12, IL-6, and TGF-β *in vitro*. Orally, *P. gingivalis* induced a local Thelper (h)1-Th2-Treg response. CIA immunizations also resulted in periodontal bone loss. 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 collagen-induced arthritis. In the complete Freund's adjuvant (CFA) model, *P. gingivalis* infection induced a greater number of osteoclasts and tissue swelling once arthritis affected the entire paw in the CFA model of disease. In the incomplete Freund's adjuvant (IFA) model for

arthritis, *P. gingivalis* increased pannus formation, bone destruction, and osteoclast numbers. Together, our results indicate that chronic oral infection with *P. gingivalis* prior to arthritis induction altered Th cell-mediated responses and increased Th17 responses in collagen-induced arthritic mice. These results are important in furthering our understanding for the potential of an oral chronic infection in altering arthritis in susceptible patients, and may have important implications for developing future preventive periodontal and RA therapies.

CHAPTER I

INTRODUCTION

BACKGROUND AND SIGNIFICANCE

Periodontal disease, *Porphyromonas gingivalis*, and host response

Periodontal disease is a chronic inflammatory disease of the periodontal tissues that affects 48% of the population aged <30 years, and is the major cause of tooth loss in adults (Albandar, 2011). It is known to be multifactorial by having contributions of the host genetic susceptibility, the presence of specific bacteria, and the influence of environmental factors in order for disease to establish and progress. *P. gingivalis* is a black-pigmented anaerobic bacterium associated with increased periodontal breakdown (Fig. 1.1). Several strains of *P. gingivalis* have been isolated from individuals with oral infections and are utilized to understand periodontal disease pathogenesis. Classically, *P. gingivalis* strains are classified as invasive or non-invasive based on differences in the ability to cause soft tissue abscesses following subcutaneous injections at distant or local sites (Neiders et al., 1989). Strains show variation in the persistence of oral colonization (Baker et al., 2000), antibody formation (Katz et al., 1996), alveolar bone loss induction, cytokine systemic responses (Marchesan et al., 2012), and prevalence in the general population (Igboin et al., 2009). In general, a shift in the

bacterial community composition occurs from supra-gingival aerobic to predominantly Gram-negative anaerobic bacteria with species that constitute the red-complex, which includes *P. gingivalis, Treponema denticola, and Tannerela fortythia* (Socransky et al., 1998). The most recent hypothesis for periodontal disease pathogenesis is called the keystone-pathogen hypothesis, which supports that certain low-abundance microbial pathogens can orchestrate disease by changing the symbiotic microbiota into a dysbiotic microbiota (Hajishengallis et al., 2012). *P. gingivalis* is a potential candidate for a keystone pathogen for disease-provoking periodontal microbiota, and therefore, is an important bacterium to study in the context of disease pathogenesis.

P. gingivalis is shown to activate several innate immune receptors, including toll-like receptor (TLR)-2, TLR-4, nucleotide-binding oligomerization domain (NOD)-2, and protease-activated receptor (PAR)-2 which ultimately contribute to disease initiation and progression (Burns et al., 2006; Chung et al., 2010; Gaddis et al., 2011). After activation of the innate immune response, it is clear that T helper (h) cells are essential for periodontal destruction (Yamaguchi et al., 2008). The majority of the literature supports that periodontal disease is driven by Th1-Th2 responses, while the role of Th17 cells has been more recently explored (Eskan et al., 2012). Several cytokines and markers related to all Th1, Th2, and Th17 cells are highly expressed in diseased gingival and periodontal tissues. It is proposed that pro-inflammatory Th1 and Th17 cells are harmful for tissue destruction, but play active roles in controlling periodontal infection, that Th2-B-cell axis actively protect the host by antibody production,

and Tregulatory (treg) cells seem to attenuate local destruction via IL-10 (Garlet, 2010). While the local immune response and pathogenesis induced by *P. gingivalis* infection is better documented, the systemic role of *P. gingivalis* has been less explored.

Several models exist to study periodontal disease in pre-clinical settings. These include the ligature model, LPS gingival injections, air-pouch model, and the gavage model. Each model provides an opportunity to investigate different steps of periodontal disease, which can be broken down into 5 discrete phases: 1) colonization, 2) invasion across the epithelium into the connective tissue, 3) stimulation of an inflammatory response, 4) induction of connective tissue breakdown and bone resorption, and 5) repair processes (Graves et al., 2012). The oral gavage consists of inoculating human strains of bacteria into the oral cavity of mice or rats in a viscous suspension of 2% carboxymethylcellulose and PBS. The most commonly used bacterium is *P. gingivalis* of several different strains. However, other periodontal bacteria have been utilized in the oral gavage model, including *A. actinomycetemcomitans* (Trombone et al., 2010), *Tanerella forsythia, and Treponema denticola* (Kesavalu et al., 2007). The gavage model has been used to mechanistically link periodontitis to atherosclerosis (Lalla et al., 2003), aortic aneurisms (Wada and Kamisaki, 2010), diabetes mellitus (Lalla et al., 1998), and RA (Cantley et al., 2011; Trombone et al., 2010). Given the chronic nature of the disease and the systemic exposure of the bacteria, the oral gavage is a suitable model to study the relationship between periodontitis and systemic conditions (Graves et al., 2012).

Periodontal Medicine and Rheumatoid Arthritis

The term periodontal medicine was defined for the first time in 1996 as a two-way relationship between periodontal disease and systemic diseases (Offenbacher, 1996). Periodontal disease is shown to be associated with several other systemic diseases, including cardiovascular disease (Friedewald et al., 2009), diabetes mellitus (Lamster et al., 2008), and rheumatoid arthritis (RA) (de Pablo et al., 2008). Accumulating clinical evidence supports an association between patients with RA and periodontal disease. RA subjects show higher severity of periodontitis and number of missing teeth compared to healthy subjects (de Pablo et al., 2008). In addition, patients with periodontitis show an RA prevalence of 3.95% compared to 0.66% prevalence in healthy controls of the same study (Mercado et al., 2000). Therefore, the relationship between periodontal disease and RA seems to be bi-directional (Fig. 1.2). The direct effect of periodontal disease in RA has been clinically shown by decreased serum erythrocyte sedimentation-rate, C-reactive protein (CRP), TNF-α levels and improved disease activity score (DAS28) after periodontal treatment in patients with both RA and periodontitis (Al-Katma et al., 2007; Erciyas et al., 2012; Ortiz et al., 2009). In pre-clinical studies, mice that received oral gavage with *P. gingivalis* prior to collagen-antibody induced arthritis developed arthritis at a greater rate compared to arthritis alone, with increased inflammation and tissue destruction (Cantley et al., 2011). While systemic cytokines were not measured in this study, an increased serum CRP was found in mice induced for both

periodontitis and arthritis compared to arthritis alone, suggesting that *P. gingivalis* was activating immune cells.

RA is an autoimmune, systemic, inflammatory condition that manifests itself mainly in multiple joints, but may affect other organs of the body. The prevalence of RA is believed to range from 0.5-1% in the population worldwide. Prevalence estimates estimated that 1.5 million U.S. adults have RA, with a higher prevalence in women compared to men (Myasoedova et al., 2010; Sacks et al., 2010). The most recent study of mortality among people with RA found a mortality ratio of 2.3 among people with RA compared to the general American population, with cardiovascular events being attributed to 40% of deaths (Wolfe et al., 1994). The etiology of RA is unknown. Studies suggest that the disease takes place as a result of an interaction between genetic factors and environmental exposures, which together lead to immune perturbation in both innate and acquired responses and subsequent chronic inflammation. Several modifiable risk factors have been studied in association with RA, including tobacco use, dietary factors, and microbial exposures. Recent studies have reported that RA patients with severe periodontitis have higher IgG- and IgM-anti *P. gingivalis* titers than non-RA controls with severe periodontitis (Smit et al., 2012). In addition, immunity to *P. gingivalis*, but not other periodontal bacteria, is significantly associated with the presence of RA-related autoantibodies in individuals at risk for developing RA (Mikuls et al., 2012). It is known that an early perturbation in the immune response occurs long before disease initiates, since studies of sera from pre-disease onset reveal the presence of antibodies against

citrullinated self-proteins and rheumatoid factors that predate disease onset by up to 10 years (Avouac et al., 2006). Therefore, furthering the understanding of early *P. gingivalis* exposure and chronic infection in arthritis development is of great importance.

Several models are utilized in the pre-clinical setting to study RA, including CIA, collagen-antibody induced arthritis, pristine-induced arthritis, zymosan-induced arthritis, methylated bovine serum albumin model, and the genetically manipulated or spontaneous arthritis models such as the TNF-α transgenic mouse, K/BxN mouse, and the Skg mouse (Asquith et al., 2009). The CIA model shares many similarities to human RA, including the breach of tolerance, generation of antibodies towards self and collagen, pathological features, and the importance of Th1/Th17 cell responses (Asquith et al., 2009; Sarkar et al., 2009). CIA is elicited in susceptible mice strains by immunization with collagen II (CII) emulsified in complete or incomplete Freund's adjuvant (CFA or IFA), which is composed of a mixture of mannide monooleate and heavy paraffin oil with or without heat-killed *Mycobacterium tuberculosis* (*M. tuberculosis*) (Brand et al., 2007). The presence of *M. tuberculosis* at the time of injection with CII and IFA is reported to facilitate arthritis development, with faster development being observed (Matthys et al., 1999).

Citrullinated proteins and inflammation

Anti-citrullinated protein antibodies (ACPA) have been recognized to be very specific for the presence of RA (Van Steendam et al., 2011). The presence

of these antibodies are also shown to be predictive for RA several years before disease onset (Bizzaro et al., 2013). ACPA family of antibodies includes anti-perinuclear factor, anti-keratin antibody, anti-vimentin, and anti-filaggrin antibody (Rosenstein et al., 2004). These proteins become antigenic through the conversion of arginine to citrulline via an enzyme known as peptidyl arginine deiminase (PAD). Deimination converts positively charged arginine residues to polar but uncharged citrullines. These sites become potential targets for IgG antibodies. The direct influence of ACPA family of antibodies in bone resorption was recently demonstrated. Antibodies against citrullinated vimentin were shown to bind to osteoclast and directly induce robust osteoclastogenesis via TNF-α expression (Harre et al., 2012). *P. gingivalis* has the capability of generating citrullinated peptides due to the presence of PAD, and is the only prokaryote that produces this enzyme (McGraw et al., 1999). It is suggested that patients with periodontal infection are exposed to antigens generated by *P. gingivalis* PAD, which become systemic immunogens and lead to intra-articular inflammation (Rosenstein et al., 2004). Clinical data shows an association between the humoral immune response to *P. gingivalis* and the presence of ACPA both in patients and free-disease relatives (Hitchon et al., 2010). Therefore, it is possible that a mechanism linking periodontal disease and arthritis is by the breach of tolerance by citrullinated peptides via *P. gingivalis*.

Osteoimmunology

Osteoimmunology is an interdisciplinary research field that investigates the interplay of the skeletal and immune systems. The majority of studies evaluate the interactions between monocytes/macrophages with osteoclasts and T cells with osteoclasts (Takayanagi, 2010). Macrophages express numerous cytokines that influence osteoclast differentiation, function, and cell survival, including IL-1β, TNF-α, IL-6, and macrophage-inflammatory protein 3-α/CCL20 (Lisignoli et al., 2007; Mori et al., 2011). Activated T cells and osteoblasts express the major cytokine that regulates osteoclast differentiation, receptor activator of nuclear factor (NF)-κB ligand (RANKL) (Kong et al., 1999). RANKL interacts with RANK, which regulates the formation of multinucleated osteoclasts from their precursors as well as their activation and survival in normal bone remodeling and in a variety of pathologic conditions. OPG protects the skeleton from excessive bone resorption by binding to RANKL and preventing it from binding to its receptor, RANK. While Th1 and Th2 cells show inhibitory effects on osteoclastogenesis via IFN-γ and IL-4, Th17 cells directly stimulate osteoclasts by inducing RANKL on osteoblasts and synoviocytes via IL-17 (Takayanagi, 2010).

In this dissertation work, in the second chapter we elucidate the systemic impact of *P. gingivalis* in periodontal disease by evaluating 3 different strains of high clinical relevance. We demonstrate that *P. gingivalis* can infect the oral cavity and maintain infection for long periods of time. We also demonstrate that the infection can alter the systemic response of mice, and that high levels of splenic Treg-related IL-10 were associated to lower amount of alveolar bone

loss. In the third chapter, we demonstrate that infection with *P. gingivalis* prior to arthritis induction with CFA increased swelling once arthritis was established in the entire paw, with increased number of osteoclasts in the joints. In the IFA model *P. gingivalis* oral infection increased the development, pannus formation, and bone loss during arthritis development. While *P. gingivalis* showed a local Th1-Th2 response, the systemic response was predominantly via Th17-related cytokines.

PROBLEM STATEMENT

Periodontal disease is shown to influence RA clinical parameters. Both diseases are known to require the innate and acquired immune response in order for disease to develop, with Th cells having a major role. Antibodies to *P. gingivalis*, but not other periodontal bacteria, are related to early onset or patients at risk for developing arthritis. With the observation that preventive treatments lead to increased quality of life and decreased treatment cost, preventive periodontal therapies would be valuable to assist patient care if periodontal disease can indeed influence arthritis. Therefore, the impact of chronic periodontitis and the important periodontal bacterium *P. gingivalis* in arthritis development and progression is important to be elucidated.

GENERAL HYPOTHESIS

We hypothesize that a chronic periodontal infection leads to a shift in cytokine expression and contributes to the development and/or progression of arthritic bone destruction via Th17 cells.

Our general hypothesis will be tested by addressing the following specific aims:

Specific aim 1: Compare the systemic cellular acquired immunological response of different strains of *P. gingivalis* using a murine chronic periodontitis model.

Specific aim 2: Determine the effect of chronic periodontal disease on immune activation during collagen-induced arthritis development in mice.

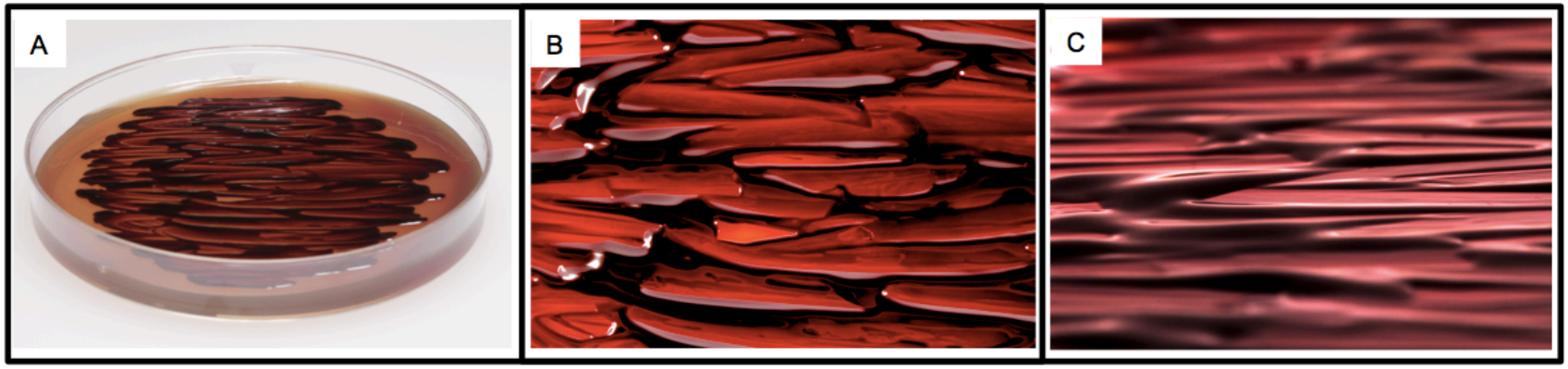

Figure 1.1. Black-pigmented bacterium *Porphyromonas gingivalis* growing in sheep-blood brucella-agar plate at **(A)** a distant view and **(B)** a closer view in 90° angle and **(C)** in 45° angle.

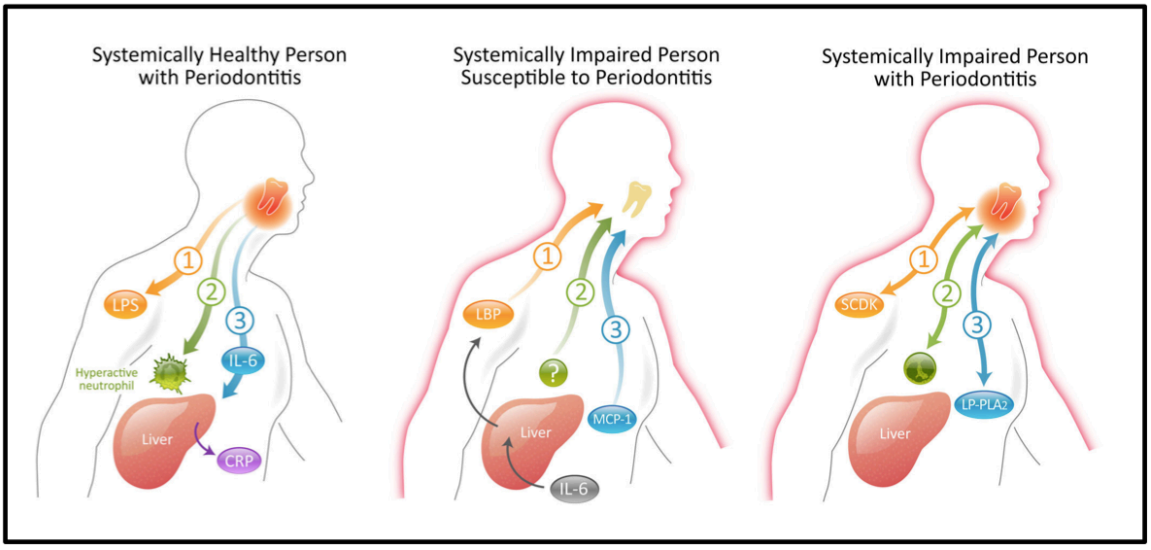

Figure 1.2. Proposed bi-directional model of the relationship between periodontitis and rheumatoid arthritis (RA). (A) Patient with periodontitis deliver components to the rest of the body influencing RA development; **(B)** Patients with RA deliver systemic components influencing periodontitis development; **(C)** Periodontitis and RA deliver systemic components and influence each other (bi-directions). Arrows indicate 3 potential mechanisms linking periodontal disease to other diseases: 1) Microbial related components that may bind to innate immune receptors, such as LPS; 2) Innate or acquired immune cellular changes, such as hyperactive neutrophils; 3) Systemic soluble factors, such as proinflammatory cytokines changes in the bloodstream. Red outline of patient depicts a systemic inflammatory condition.

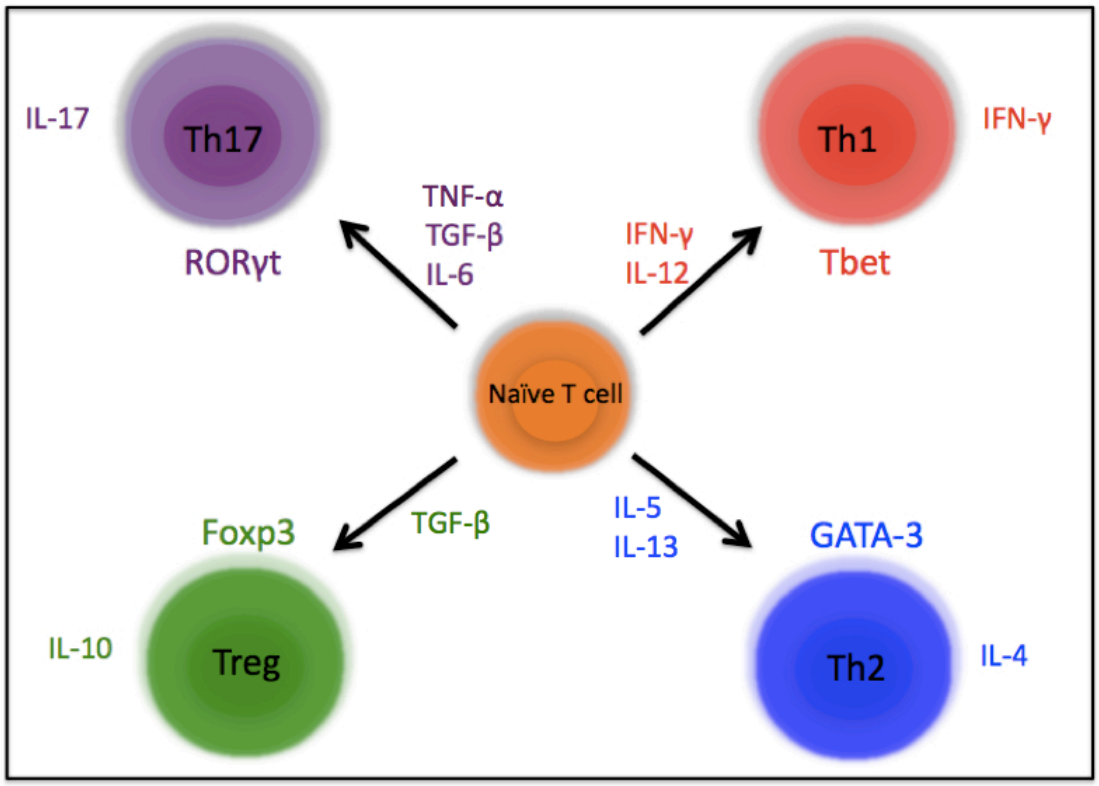

Figure 1.3. T cell-related cytokines hypothesized to be implicated in periodontitis and arthritis development. Based on the presence of cytokines in the local environment, naïve T cells will differentiate into different subsets of T cells. The presence of TNF-α, and TGF-β with IL-6 leads to expression of the transcription factor RORγt and Th17 cell polarization, which expresses IL-17; presence of IFN-γ and IL-12 leads to expression of the transcription factor T-bet and Th1 polarization, which expresses IFN-γ; TGF-β will lead to transcription factor Foxp3 expression and a Treg cell development, which expresses IL-10; the presence of IL-5 and IL-13 leads to expression of transcription factor GATA-3 and to a Th2 cell development and expression of IL-4.

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CHAPTER II

Divergence of the systemic immune response following oral infection with distinct strains of *Porphyromonas gingivalis*

ABSTRACT

Introduction: Periodontitis is a polymicrobial oral infection characterized by the destruction of tooth supporting structures that can be linked to systemic diseases such as cardiovascular disease, diabetes, or rheumatoid arthritis. *Porphyromonas gingivalis*, a bacterium implicated in the etiology of periodontitis, has shown variation in inducing T-cell responses among different strains. Therefore, in this study we investigated the strain specific immune response using a murine experimental model of periodontitis.

Material and Methods: Periodontitis was induced by *P. gingivalis* strains A7A1-28, W83, and W50, and later confirmed by *P. gingivalis* presence in the oral microflora and alveolar bone resorption. Splenocytes were evaluated for gene expression, cellular proteins, and cytokine expression. Dendritic cells were stimulated *in vitro* for cytokines involved in Th subset differentiation.

Results: The results showed that *P. gingivalis* had the ability to alter the systemic immune response after bacterial exposure. Strains W50 and W83 were shown to

induce alveolar bone loss, while mice that received A7A1-28 strain did not significantly promote bone resorption. Splenocytes derived from mice infected with strains W50 and W83 induced expression of high levels of IL-4, while A7A1-28 stimulated increased IL-10. Stimulation of dendritic cells *in vitro* showed a similar pattern of cytokine expression of IL-12p40, IL-6, and TGF-β among strains.

Conclusion: A distinct systemic response *in vivo* was observed among different strains of *P. gingivalis,* with IL-10 associated with the least amount of alveolar bone loss. Evaluation of pathogen-driven systemic immune responses associated with periodontal disease pathogenesis may assist in defining how periodontitis may impact other diseases.

INTRODUCTION

Periodontal disease is an immune-inflammatory infection of the tooth supporting structures and a major cause of tooth loss among the adult population. Increasing evidence shows an association between periodontal disease and other diseases, including cardiovascular disease (Friedewald et al., 2009), diabetes mellitus (Lamster et al., 2008), and rheumatoid arthritis(de Pablo et al., 2008). While this association seems to be bi-directional, studies show that periodontitis alone can be considered a modifying factor for human diseases (Garlet, 2010). Still, the mechanism causing this effect is not well defined and the systemic immunological influence of periodontal disease is not clearly understood.

The acquired immune response is known to be important for periodontal disease development (Garlet, 2010). Specific microbial components that activate antigen-presenting cells, such as dendritic cells, will lead to production of a set of cytokines. The pattern of cytokines expressed determines subsequent polarization of a distinct antigen-specific lymphocyte response. Characteristic markers of T helper (Th) 1, Th2, Th17, and regulatory T cells (Treg) subsets have all been described in diseased periodontal tissues (Garlet, 2010). Therefore, the exact crosstalk that occurs among Th cytokines in periodontal disease and its impact on disease outcome is still to be determined. However, it is clear that Th cells are essential for periodontal destruction. The absence of B cells does not impede LPS-induced bone resorption (Yamaguchi et al., 2008). But B cells also seem to contribute to periodontal disease development, since B-cell deletion prevented alveolar bone loss in mice after infection with *Porphyromonas gingivalis* (Baker et al., 2009).

P. gingivalis is a pathogenic bacterium associated with increased risk to periodontal breakdown and disease recurrence (Socransky et al., 1998; Socransky et al., 2002). Colonization by *P. gingivalis* can cause changes in the amount and composition of the oral commensal microbiota by perturbing the host immune response (Hajishengallis et al., 2011). Several strains of *P. gingivalis* have been isolated from individuals with oral infections and are utilized to understand periodontal disease pathogenesis. Some studies have directly compared the biological differences induced among *P. gingivalis* strains in animal models. Classically, *P. gingivalis* strains are classified as invasive or non-

invasive strains based on differences in the ability to cause soft tissue abscesses following subcutaneous injections at distant or local sites (Neiders et al., 1989). In addition to soft tissue abscesses, different strains vary in their ability to cause periodontal bone loss (Baker et al., 2000), serum and saliva antibody expression (Katz et al., 1996), and death in mice (Ebersole et al., 1995) and drosophila (Igboin et al., 2011). While only 36% of mice gavaged with strain A7A1-28 died, W50 strain caused death in 100% of the animals in a 48h period (Katz et al., 1996), demonstrating the different pathogenic potential of these strains. In addition, the induced pattern of cytokines driving the development of Th cells *in vitro* has also shown differences among strains (Gemmell et al., 2002). Therefore, we speculate that the systemic effect following an oral infection with different *P. gingivalis* strains is also distinct. In this study, we established periodontal infection in mice with the strains A7A1-28, W83, and W50, and hypothesized that, along with the differences found in the amount of alveolar bone loss established, *P. gingivalis* would induce a distinct pattern of systemic immune responses among strains. The *P. gingivalis* strains chosen in this study are among the predominant ones isolated from clinical samples and are utilized as inducers of periodontitis in pre-clinical models of disease (Igboin et al., 2009).

MATERIALS AND METHODS

In order to evaluate the systemic immune response caused by different *P. gingivalis* strains, we tested strains A7A1-28, W83, and W50 both *in vivo* and *in vitro*. All animal experiments were approved by the Institutional Animal Care and

Use Committee of the University of Michigan (Ann Arbor, MI) and conformity to ARRIVE guidelines for preclinical studies. *In vivo*, DBA/1J mice (The Jackson Laboratory, Bar Harbor, Maine) were infected with the 3 different strains and followed during 42d for periodontal disease development (Fig 2.1). Periodontal disease was confirmed by the presence of *P. gingivalis* in the oral cavity by PCR and the stimulation of bone loss assessed by micro-computed tomography and histomorphometric analysis. In addition, splenocytes were collected and evaluated by flow-cytometry and gene expression, as well as treated with PMA/Ionomycin and evaluated for protein expression by ELISA. *In vitro*, bone-marrow derived dendritic cells from non-infected mice were isolated and activated with different *P. gingivalis* strains, and later evaluated for protein expression by ELISA.

Bacterial strains

P. gingivalis strains A7A1-28, W83, and W50 were grown under anaerobic conditions (85% N_2, 5% CO_2, and 10% H_2) on pre-reduced brucella broth agar (BD Biosciences, San Jose, CA) plates enriched with 5% sheep blood (Remel, Lenexa, KS), 5mg/mL hemin, and 1 ug/mL menadione (Sigma-Aldrich, St Louis, MO). *P. gingivalis* colonies were confirmed by PCR of Arg-gingipain (201 bp) and DNA sequencing before experiments were performed. Before PCR analysis was performed, the samples were heated at 100°C for 30 min. The following primer was utilized: 5'-CAAAATTACTCCGGGGATCA-3' (forward) and 5'-GCCGAAGCAATACAAAGAGC-3' (reverse). New bacterial passages were

further confirmed by the presence of homogeneous black-pigmented colonies and/or BANA test positivity (Loesche et al., 1992).

Murine periodontitis model

Thirty-two DBA/1J male mice (4 weeks old) were divided into 4 groups (8 mice/group) that were infected with either A7A1-28, W83, W50, or vehicle. Mice were given sulphamethoxazole at 0.87 mg/mL and trimethoprim at 0.17 mg/mL (Hi-Tech Pharmacal Co. Inc., Amityville, NY) in mili-Q water *ad libitum* for 10d, followed by 3d without antibiotics. For infection, *P. gingivalis* was suspended at 10^{10} CFU/mL as determined by optical density of 600 nm. Mice were inoculated with 10^9 CFU of bacteria in 100uL of PBS with 2% carboxymethylcellulose (CMC; Sigma-Aldrich, St Louis, MO) by oral gavage at 6 separate periods, with 2d intervals as previously described (Baker et al., 1999; Sasaki et al., 2004). The vehicle group received CMC alone. Mice were weighed at study initiation and at sacrifice (Fig. 2.1).

Oral microflora analysis

For *P. gingivalis* colonization analysis, the oral microflora was collected at baseline and 1 d, 7 d, 14 d, 21 d, 28 d, and 42 d after the last gavage. A sterile swab was placed in the oral cavity of mice and swirled for 15s, followed by its placement in 1mL PBS. Bacterial infection was confirmed by PCR of Arg-gingipain. In order to identify the minimum amount of bacteria detectable in the oral microflora of mice, PCR analysis was performed with *P. gingivalis* at the

following concentrations: 0, 10^2, 10^3, 10^4 CFU/mL. PCR products were evaluated by electrophoresis and densitometry by ImageJ software (http://rsb.info.nih.gov/ij/download.html).

Micro-computed tomography (micro-CT)

Mice were sacrificed at 42d following gavage and subsequently maxillae were harvested and immediately fixed in 10% formalin for 24h, and transferred to 70% alcohol. Fixed, non-demineralized mouse maxillae were scanned using a Pxs5-928EA cone-beam micro-CT system for the generation of 3-D images (GE Healthcare, Little Chalfont, UK). Analysis was performed by a calibrated masked examiner (SL). All micro-CT maxillary images were reoriented with morphological landmarks identified as previously described (Park et al., 2007). All three planes were identified by morphological landmarks to ensure consistent orientation. The region of interest (ROI) was then chosen to include as much interproximal alveolar bone while excluding tooth structures. Two transverse plane boundaries of ROI were chosen between the M1 root furcation and the root apex of M1 and M3. Tissue mineral content, bone volume, bone volume fraction, and bone mineral content were obtained using bone analysis command of GEHC MicroView Analysis Plus software (GE Healthcare, Little Chalfont, UK), as previously described (Park et al., 2007).

Histological preparation and analysis

After analysis by micro-CT, maxillae were decalcified in EDTA (Acros, New Jersey, USA) for a period of 14d and then embedded in paraffin. Saggital sections (4-5 μm thick) were obtained from each maxilla at the molar region of M1, M2, and M3 for a 2-D evaluation. Coded sections were stained with hematoxylin and eosin (H&E) for histomorphometric analysis, further performed by a masked and calibrated examiner (JM). The distance between the cementum-enamel junction (CEJ) and alveolar bone crest (AB) in the mesial and distal region of M1 with mesial of M2 were measured using NIS Elements Basic Research imaging software (Nikon Inc., Melville, NY) (Park et al., 2011).

Spleen harvest and processing

After mice were sacrificed, spleens were removed, segmented, and placed in 10mL serum-free RPMI medium 1640 (Gibco, Life technologies, Grand Island, NY). Cells were dispersed through a 70 μm cell strainer into a 50mL conical tube and centrifuged at 480 RCF for 3 min. The pellet was suspended in 5mL of ACK lysis buffer (155mM NH4Cl, 10mM KHCO3, 0.1mM EDTA) for 5 min to allow for lysis of red blood cells. Serum-free medium was added to halt lysis; supernatant was collected and centrifuged at 480 RCF for 5 min in order to pellet the leukocytes. The total number of splenocytes was calculated and further experiments were performed.

Quantitation of splenocyte mRNA expression

Processed splenocytes derived from mice at 42d after gavage were treated with 1mL TRIzol reagent (Invitrogen, Rockville, MD, USA) and stored at -80°C for mRNA expression and further analysis. RNA was isolated via the TRIzol method, and total RNA was quantified by spectrophotometry. Single-stranded cDNA was systhesized from 0.5 ug RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). cDNA was amplified via TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA). The following transcription factors were evaluated: T-bet for a Th1-driven response, GATA-3 for a Th2-driven response, RORγt for a Th17-driven response, Foxp3 for a Treg-driven cell response, and FasL for a killer cell response. Amplification was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Carlsbad, CA). Gene expression was normalized with the housekeeping gene GAPDH and relative quantification of the data generated was carried out using the comparative CT method (Schmittgen and Livak, 2008).

Flow cytometric analysis

Splenocytes were isolated and labeled with either anti-mouse CD3-phycoerythrin (PE)-cychrome 7 (Cy7) conjugate (BD Biosciences, San Jose, CA) and anti-mouse CD4-fluorescein isothiocyanate (Biolegend, San Diego, CA) for T cells or B220-PE-Cy7 conjugate, CD5- allophycocyanin (APC), and FasL-PE (BD Biosciences, San Jose, CA) for killer B cells, followed by in FixPerm buffer (Biolegend, San Diego, CA) fixation overnight at 4°C. Fixation solution was washed thoroughly followed by labeling of cells with FoxP3-PE, IL-17-APC, or IL-

10-PE (EBiosciences, San Diego, CA) for T cells. Flow cytometry was performed on a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA), and cell marker analysis was done using FloJo software (TreeStar Inc., Ashland, OR).

Splenocyte cultures for analysis of cytokine expression

Splenocytes were plated in 12-well plates at 5 x 10^6 cells/mL in RPMI media 1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (PS) (Gibco, Grand Island, NY), 1% HEPES (Gibco, Grand Island, NY), 1% sodium pyruvate (HyClone, South Logan, Utah), and 0.1% 2-Mercaptoethanol (Gibco, Grand Island, NY). Cells were treated with phorbol 12-myristate 13-acetate (PMA)/ionomycin (Sigma-Aldrich, St Louis, MO) for B and T cell activation. PMA/ionomycin mimics antigen receptor activation and elicits cytokines from previously activated lymphocytes, with usually higher degrees of expression compared to antigen reactivation. Cell-free supernatants were collected after 48h treatment from cultures and evaluated by ELISA for interferon (IFN)-γ for Th1-driven response, IL-17 (BD Biosciences, San Jose, CA) and IL-6 (R&D systems, Minneapolis, MN) for Th17-driven response, IL-10 and TGF-β (R&D systems) for Treg response, IL-4 (R&D systems) for Th2 cell response according to the manufacturer's protocol.

Dendritic cell activation

In order to evaluate the cytokine production from antigen-presenting cells, bone-marrow derived dendritic cells were isolated as previously described and

activated with different strains of *P. gingivalis (Lutz et al., 1999)*. Briefly, murine hind limbs from non-infected DBA/1J male mice were collected. Bone marrow was flushed with RPMI supplemented with 10ug GM-CSF, 10% fetal bovine serum, 1% PS, and 0.1% 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO). In order to obtain dendritic cells with high percent purity, the flushed bone-marrow was cultivated for 10 d and plated at 10^5 cells/well. Cells were treated with the different strains of *P. gingivalis* at multiplicity of infection (MOI) of 0.1, 1, and 10 for 1h, followed by treatment with gentamycin at 50ug/mL. Supernatants were collected after 18h for cytokine expression analysis. In order to evaluate the immune response driven by *P. gingivalis* infection, cytokine expression was evaluated by ELISA for IL-12p40, IL-6, and TGF-β (BD Biosciences, San Jose, CA) for Th1 and Th17 activation, and Treg response, respectively.

Statistical analysis

The preclinical studies consisted of eight animals/group at the 42d analysis. Dendritic cell experiments were performed in triplicates. The differences among groups were statistically assessed by ANOVA (significance defined as $p<0.05$), with Tukey multiple comparison post hoc test. Group-infected mice were compared to vehicle alone by t-test. Data were analyzed by GraphPad Prism 5.0 program (GraphPad Software, La Jolla, CA).

RESULTS

Periodontal disease establishment and development

P. gingivalis colonies were confirmed by PCR of Arg-gingipain (201 bp) (Fig. 2.2A) and DNA sequencing. In order to evaluate the amount of *P. gingivalis* CFU necessary to be present in the oral microflora of mice for detection by PCR of Arg-gingipain analysis, known amounts of *P. gingivalis* were added to mouse oral samples at 0, 10^2, 10^3, 10^4 CFU. While the presence of 10^2 CFU of *P. gingivalis* was not detectable in the oral microflora by PCR, the presence of 10^3 and 10^4 CFU was sufficient for *P. gingivalis* detection (Fig. 2.2B). At baseline prior to oral gavage, we evaluated the oral microflora by PCR of Arg-gingipain, and all mice were negative for the presence of *P. gingivalis* (Fig. 2.2C). PCR evaluations of the oral samples demonstrated *P. gingivalis* infection at all time points evaluated. At 42d after the last gavage was performed, 22/24 mice were infected with *P. gingivalis* (Fig. 2.2D-F). Only one mouse from groups W83 (Fig. 2.2E) and W50 (Fig. 2.2F) failed to demonstrate *P. gingivalis* confirmation at the end of the experiment. When evaluated by band intensity, W83 showed the highest amount of recovery from the oral cavity, followed by A7A1-28 and W50 strains. None of the mice in the vehicle group was positive for *P. gingivalis*. No differences were found in mice weight gain among groups, with a mean 44.46% weight gain.

For alveolar bone analysis by micro-CT (Fig. 2.3A), all four bone-related parameters evaluated showed a significant difference among groups (ANOVA $p<0.05$). For tissue mineral content, bone volume, and bone mineral content, a statistical difference was observed between groups W50 and vehicle (Tukey $p<0.05$). A higher loss was observed in W50-infected animals by tissue mineral

content measurements with 14.1% loss (Fig. 2.3B), followed by 11.6% loss by bone volume analysis (Fig. 2.3C), and 11.4% loss of bone mineral content (Fig. 2.3D) compared to vehicle group. Mice gavaged with W83 strain presented statistically significant differences in bone loss compared to vehicle group (Tukey $p<0.05$) when evaluated for tissue mineral content with 14.3% loss (Fig 2.3B), and for bone volume fraction with 7.9% loss (Fig. 2.3E). A7A1-28-infected animals showed a trend of bone loss compared to vehicle among all parameters with a tissue mineral content loss of 6.6%. However, these differences did not reach statistical significance. The vehicle group consistently showed the highest volumetric amount of alveolar bone among all groups when evaluated by all four parameters.

Histomorphometric analysis performed in the mesial region of M1 showed that the distance between the AB to the CEJ was significantly higher in W83-infected animals when compared to vehicle (Tukey, $p=0.008$) followed by A7A1-28-infected animals and W50-infected animals (Fig. 2.4). Measurements performed in the interproximal region of M1 and M2 showed W50 with the greatest degree of bone loss (103.47±33.2 μm), followed by A7A1-28 (94.23±25.7 μm), W83 (90.8±24.9 μm), and vehicle (82.6±20.4 μm).

Systemic Immune Response

At 42d post-gavage, splenocyte numbers were similar among groups with an average of 8.9×10^7 cells/mouse. Non-activated splenocytes from control and *P. gingivalis*-infected mice did not show differences in the mRNA expression of

the transcription factors T-bet, GATA-3, RORγt, Foxp3, or FasL (data not shown). In addition, $CD3^+CD4^+$ non-activated T cells were similar among groups when intracellular staining for Foxp3 and IL-10 for a Treg response or FasL expression for a killer cell response was evaluated. Still, a trend towards an increase of CD3+CD4+Foxp3+ cell percentage was observed in *Pg*-infected mice (Fig. 2.5). However, a higher percentage of CD3+CD4+Foxp3+ was observed in grouped infected-mice compared to vehicle-treated mice alone ($p=0.019$). When splenocytes were evaluated for the expression of $CD5^+B220^+$ cells, a higher percentage was found in mice gavaged with W83 compared to all the other groups ($p<0.0001$) (Fig. 2.5D).

Interestingly, splenocytes stimulated with 1% PMA/Ionomycin showed a distinct pattern of cytokine expression (Fig. 2.6). Mice gavaged with the A7A1-28 strain had a significantly higher expression of IL-10 compared to vehicle and mice gavaged with W83 strain ($p=0.0006$). A higher expression of IL-4, was found in both W83 and W50 groups with a significantly lower expression in the A7A1-28 group and vehicle ($p=0.04$). The expression of IFN-γ was significantly lower in the W83 -infected animals when compared to either vehicle or the A7A1-28 -infected animals ($p=0.01$). No differences were found among groups for the expression of the cytokines IL-17, IL-6, or TGF-β (Fig. 2.6).

Innate Immune Response

Dendritic cells stimulated in vitro with the three strains of P. gingivalis showed expression of IL-12p40, IL-6, and TGF-β, in a dose-dependent manner with no differences observed among strains (Fig. 7).

DISCUSSION

An estimated 48.2% of American adults have periodontal disease, which is the major cause of tooth loss among adults (Lutz et al., 1999). *P. gingivalis* is an important bacterium involved in disease pathogenesis by inducing a local chronic host inflammatory response that leads to alveolar bone destruction. Our study demonstrated multiple pathogenic differences among different *P. gingivalis* strains. This could be important for understanding the relationship between oral and systemic diseases affected by *P. gingivalis* infection. A difference in both alveolar bone resorption and the systemic acquired immune response was found among different strains of *P. gingivalis*. Mice gavaged with strains W50 and W83 had a predominant systemic IL-4 response (Fig. 2.6D) and higher amount of local alveolar bone loss compared to vehicle (Fig. 2.3). Mice gavaged with strain A7A1-28 had a predominant systemic production of IL-10 and IFN-γ (Fig. 2.6) with less induction of local alveolar bone loss (Fig. 2.3). Interestingly, the genetically closely related strains W83 and W50 displayed more similar results, while strain A7A1-28 that shows a divergence in the genome of 3.5% and a correspondingly more distinct response (Igboin et al., 2009).

It is interesting to note that the absence of a significant local oral response observed by strain A7A1-28 strain did not translate into a lack of an *in vitro* or systemic response. While strain A7A1-28 did not induce significant amount of bone loss, it resulted in potent dendritic cells responses *in vitro*, as well as in IL-17 induction. Interestingly, strain W83 and W50 had induction of significant oral bone loss but less splenic cytokine expression. Based on the results, we speculate that it would be interesting to sub-divide patient samples based on *Pg* strains when evaluating systemic effects of periodontitis.

IL-10 is an important immune regulatory cytokine that has been shown to regulate both IFN-γ and IL-4 driven immune responses (Albandar, 2011; Hoffmann et al., 2000). IL-$10^{-/-}$ mice show severe alveolar bone loss when infected with *P. gingivalis (Sasaki et al., 2004)*. Kobayashi *et al.* showed that gingival mononuclear cells stimulated with PMA/Ionomycin *in vitro* had IL-10 production up-regulated 30d after *P. gingivalis* infection with strain 33277, which was not observed at the early time points of 1, 7, and 15d (Kobayashi et al., 2011). Importantly, IL-10 polymorphisms that may be associated with differing levels of IL-10 expression are related to periodontal disease progression clinically (Cullinan et al., 2008). In alignment with our findings, these studies suggest that IL-10 is an important molecule in the balance between inflammatory, humoral, and infection with *P. gingivalis*.

Since mice gavaged with strain A7A1-28 displayed less bone loss and higher expression of IL-10 from re-stimulated spleen cells, we investigated whether regulatory T and B cells were distinctively up-regulated in the spleen. All

strains of *P. gingivalis* showed a trend toward induction of splenic $Foxp3^+$T cells (Fig. 2.5A). Unexpectedly, A7A1-28 group had the lowest percentage of $CD5^+B220^+$ cells (Fig. 2.5D), a population of B cells reported to produce IL-10 (Haas et al., 2005; O'Garra and Howard, 1992; Yanaba et al., 2008). $CD5^+$ B cells have also been demonstrated to express FasL in the presence of IL-4 and IL-10, and to mediate T cell death in an arthritic model (Lundy and Fox, 2009). Despite differences in IL-10, IL-4 and $CD5^+$ B cells, we found no difference among groups in the mRNA expression of FasL (Fig. 2.5C). Therefore, the higher IL-10 expression from splenocytes derived from mice gavaged with A7A1-28 strain could not be directly attributed to either Breg or Treg cells. Further, no change in splenic FasL expression could be attributed to *P. gingivalis* infection. One possibility is that the expression of IL-10 from splenocytes could be attributed to $Foxp3^-$ cells, such as the Tr1 subset. However, this strain does not express a definite prototypic surface marker for exact identification (Pot et al., 2011).

The importance of the acquired immune response locally in periodontal bone loss has been demonstrated by reduced bone loss in mice lacking both B and T cells (Baker et al., 2009; Yamaguchi et al., 2008). *P. gingivalis* is an important bacterium commonly found in patients with periodontal disease, and is associated with tissue breakdown and disease recurrence (Socransky et al., 2002). *P. gingivalis* strains A7A1-28, W83, and W50 evaluated in this study are among the most prevalent strains found worldwide (Igboin et al., 2009), and are therefore clinically relevant. The mouse DBA1/J is a strain genetically suitable for

induction of inflammatory diseases, including arthritis, and parallels the role of genetic loci as an important risk factor for human rheumatoid arthritis. The susceptibility of DBA/1J mice to arthritis is based on the MHC Class II loci and unique ability to present arthritis-associated antigens to T helper cells. To our knowledge, there have been no studies published that suggest a different response of these mice to toll-like receptor antigens, such as *P. gingivalis*. Because rheumatoid arthritis has been associated to periodontal disease in patients (de Pablo et al., 2008), the systemic effect of *P. gingivalis* in this strain of mice is of importance. We utilized animals at 4-weeks of age to allow for the development of a co-disease model that considers a younger set of animals.

When *P. gingivalis* was evaluated for its presence in the oral microflora by PCR analysis, W83 showed higher band intensities, followed by strain A7A1-28 and W50 (Fig. 2.2). Similar to our findings, recovery of W50 strain from the oral cavity of rodents using direct bacteria cultivation was lower when compared to strain A7A1-28, with only 17% being recovered by 42d in one of the studies (Baker et al., 2000; Katz et al., 1996). Still, in accordance with Baker *et al.,* the differences found in the infection assessment did not seem to influence the amount of alveolar bone loss induced by the W50 strain. Our results differ, however, in the amount of alveolar bone loss observed at the end of the experiment by different strains. Baker *et al.* (Baker et al., 2000) found that strain A7A1-28 and W50 induced similar amounts of bone loss, while in our study A7A1-28 strain induced minimal alveolar bone loss (Fig. 2.3). This finding could perhaps be explained by the different methods utilized in the studies to assess

the alveolar bone level or differences between strains of mice. Although not directly comparable, our micro-CT and histomorphometry data were quite congruent. The histomorphometry is a classical method for evaluating bone loss and provides histologic visualization of the inflammatory process (in 2-D), but only provides analysis of a small section of the entire bone loss induced. The micro-CT method provides a 3-D analysis of the bone loss that occurs by evaluating the quality of the entire maxillae that is present and is more comprehensive.

It is important to compare data generated *in vitro* to *in vivo* results to determine the degree to which *in vitro* generated cells truly reflect the biology of responses generated under physiological, more complex conditions. Our results show that while non-activated splenocytes from control and *P. gingivalis* infected mice did not show differences in the expression of transcription factors, splenocytes that were reactivated showed a strong distinct response. This demonstrates that even though there was not an active immune response happening in the spleen, the exposure to *P. gingivalis* had a systemic effect demonstrated by the ability to change the profile of splenic cytokine expression upon cell reactivation. While the dendritic cells stimulated *in vitro* had similar responses among strains, with expression of IL-12p40, IL-6 and TGF-β (Fig. 2.7), the *in vivo* scenario was distinct (Fig. 2.6). A potential explanation for the differences found between the *in vitro* and systemic *in vivo* results are the oral microflora changes that occur when *P. gingivalis* is present, an alteration called dysbiosis (Hajishengallis et al., 2012). Dysbiosis has the potential to impact the

systemic immune response and shown to be implicated in a variety of diseases, including obesity, metabolic disorders, and inflammatory bowel disease (Hill and Artis, 2010; Garret et al 2010). Therefore, while the *in vitro* data is a direct result of the effect of *P. gingivalis* in immune cells, the *in vivo* data could be the result of changes that occur in the amount and composition of the oral microbiota due to *P. gingivalis* colonization. Still, in accordance with our results, Vernal *et al.* also did not find differences in the pattern of cytokines expressed by dendritic cells stimulated with different serotypes of *P. gingivalis in vitro* (Vernal et al., 2009), with all strains showing expression of IFN-γ, IL-1β, IL-12, TNF, IL-6, and IL-10. However, we did not observe a higher response to W83 when compared to A7A1-28 (Fig. 2.7). This could be related to the differences found in mRNA versus protein expression. When the *in vivo* T cell activation was evaluated, animals gavaged with *P. gingivalis* strain 33277 displayed stimulated splenocytes expressing high levels of IFN-γ, IL-2, IL-10, and IL-4, which was consistent with a mixed Th1 and Th2 response (Katz et al., 1996). Co-culture experiments using dendritic cells and T cells also suggest a mixed Th1 and Th2 response (Jotwani and Cutler, 2004; Katz et al., 1996). In agreement with our data, these studies suggest that the Th cell type driving the *in vivo* response of *P. gingivalis* is more complex than what can be observed with single cell isolates in the *in vitro* environment. The spleen is a secondary lymphoid organ that contains approximately 55% B cells, 35% T cells, and 10% of other leukocytes, including macrophages and natural killer cells. The spleen is a site of interaction between

elements of both the innate and adaptive immune response and is, therefore, an important organ to be evaluated in the context of systemic exposures to bacteria.

In summary, *P. gingivalis* had the ability to alter the systemic immune response after bacterial exposure. Strains W50 and W83 were shown to induce alveolar bone loss, while mice that received A7A1-28 strain did not significantly promote bone resorption. Splenocytes derived from mice infected with strains W50 and W83 induced expression of high levels of IL-4, while A7A1-28 stimulated increased IL-10. Stimulation of dendritic cells *in vitro* showed a similar pattern of cytokine expression of IL-12p40, IL-6, and TGF-β among strains. A distinct systemic response *in vivo* was observed among different strains of *P. gingivalis,* with IL-10 associated with the least amount of alveolar bone loss. Evaluation of pathogen-driven systemic immune responses associated with periodontal disease pathogenesis may assist in defining how periodontitis may impact other diseases.

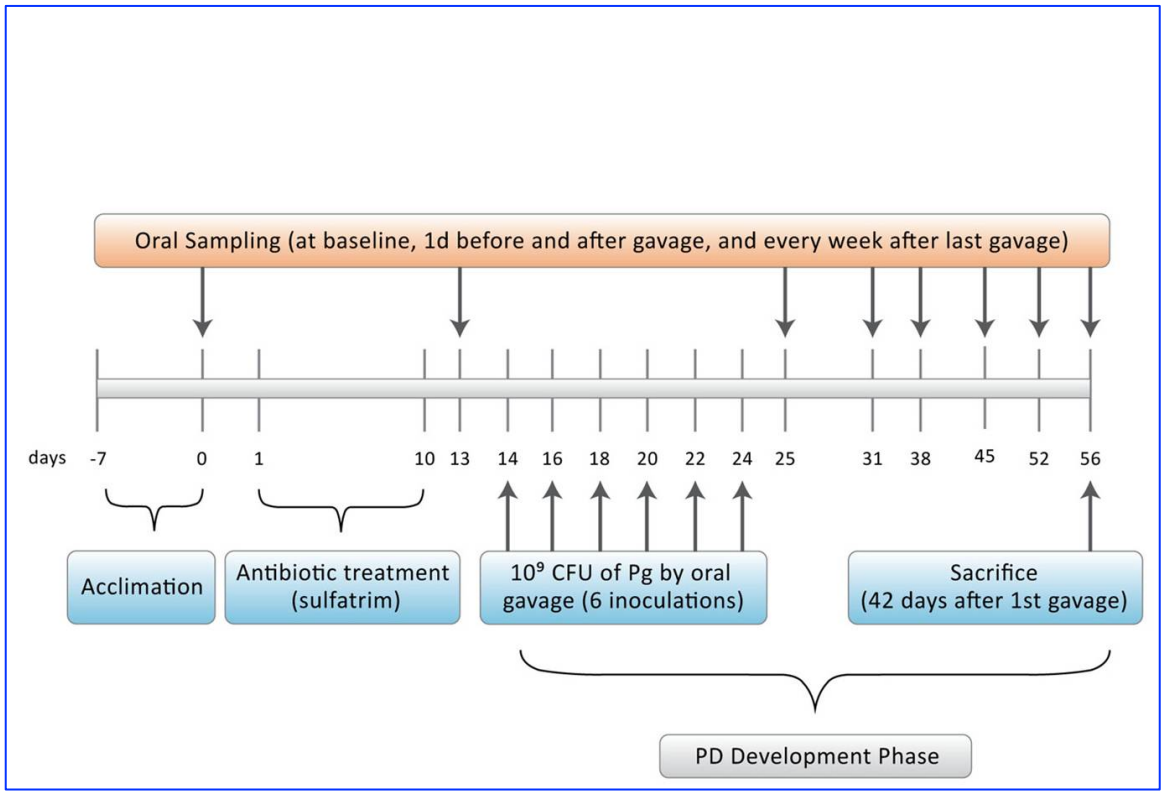

Figure 2.1. Schematic diagram illustrating the study experimental design. This timeline displays the *in vivo* experiment, including mice acclimation, antibiotic treatment, *P. gingivalis* infection period and periodontitis developmental phase, oral microflora sampling, spleen harvest, and sacrifice.

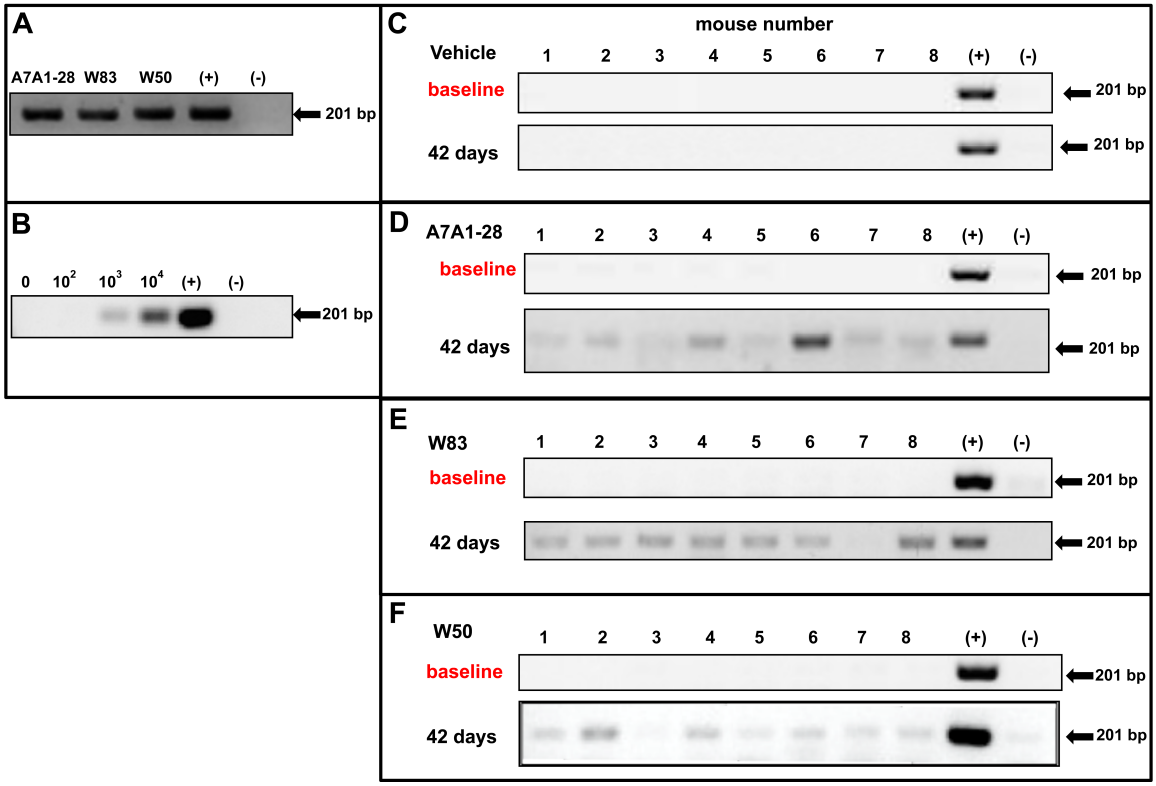

Figure 2.2. PCR analysis of *P. gingivalis* oral infection determined by Arg-gingipain (201 bp). (**A)** Confirmation of *P. gingivalis* colonies of strains W83, W50, and A7A1-28 before gavage. (**B)** *P. gingivalis* CFU at 0, 10^2, 10^3, 10^4 were added to a sample collected from the oral microflora of mice to determine the detection limit via PCR**.** Oral microflora analysis: samples of mice infected with *P. gingivalis* at baseline (upper panel) and at 42d after the first gavage (lower panel). Numbers 1 to 8 represent individual mice gavaged with **(C)** vehicle alone, **(D)** A7A1-28, **(E)** W83, and **(F)** W50.

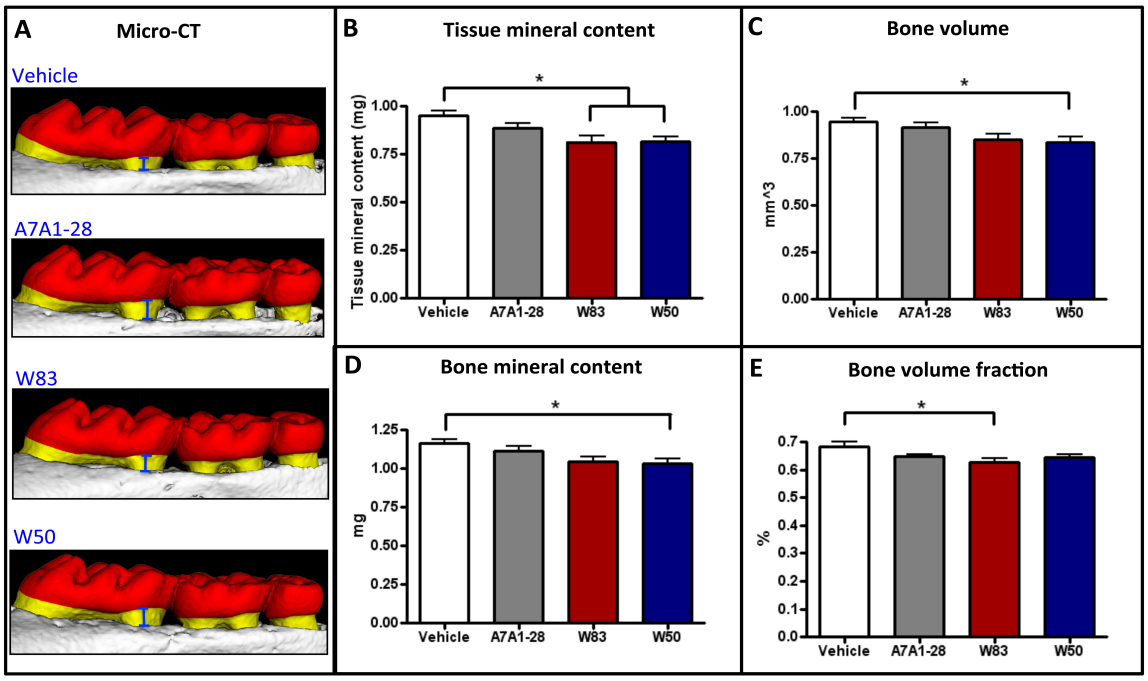

Figure 2.3. Alveolar bone loss measured by micro-computed tomography. (**A)** Representative images of the alveolar bone loss found in mice at 42d after gavage was performed with the 3 different strains A7A1-28, W83, and W50. **B**. Analysis performed for tissue mineral content, bone mineral content, bone volume, and bone volume fraction. Error bars indicate standard error. Asterisks denote differences found among groups by Tukey test ($p<0.05$).

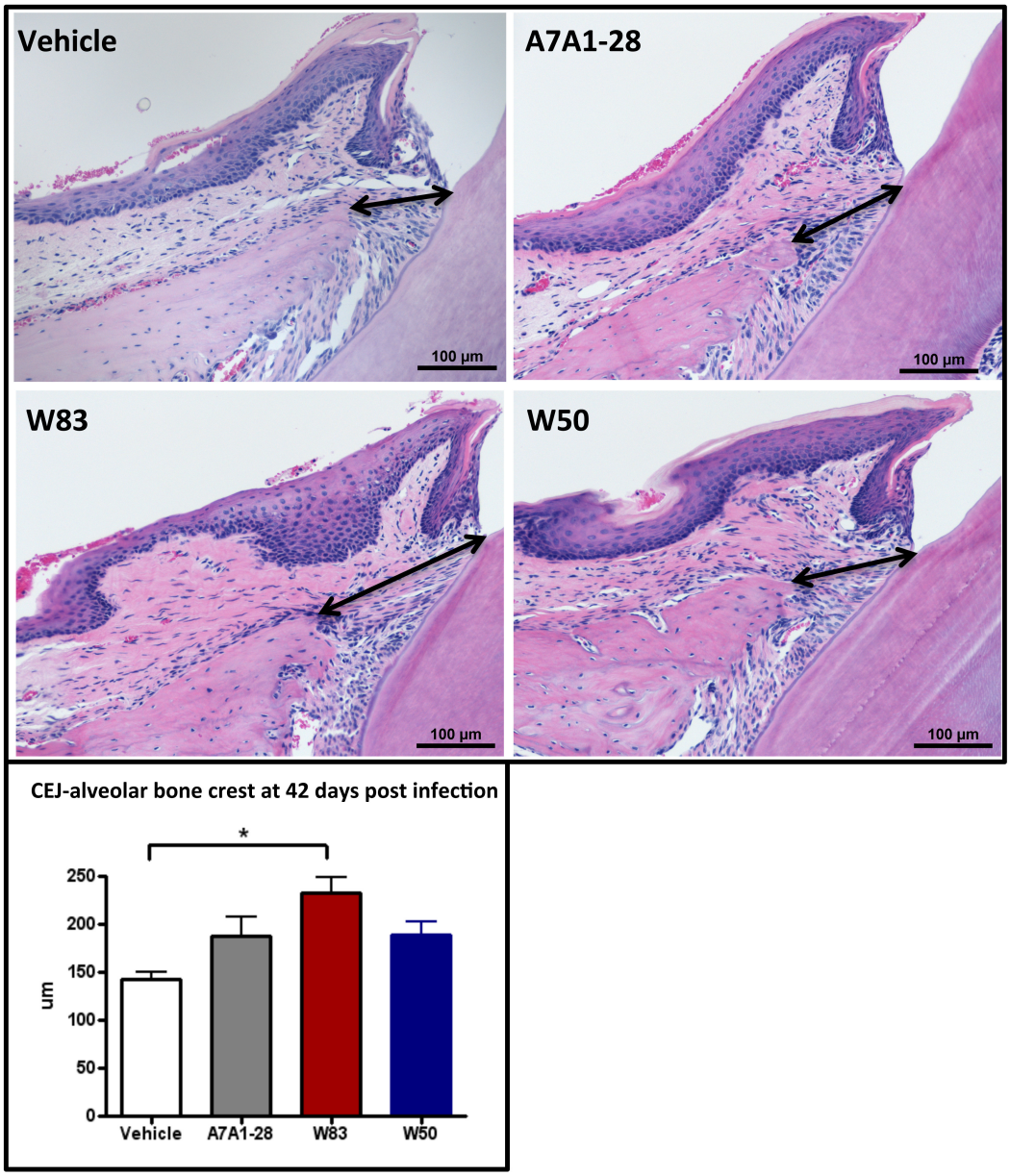

Figure 2.4. Histomorphometric image analysis performed in maxillae sections of mice at 42d after gavage; the distance between the AB to the CEJ in M1 was measured in micrometers (depicted with black arrows) (H&E stain, 20X).

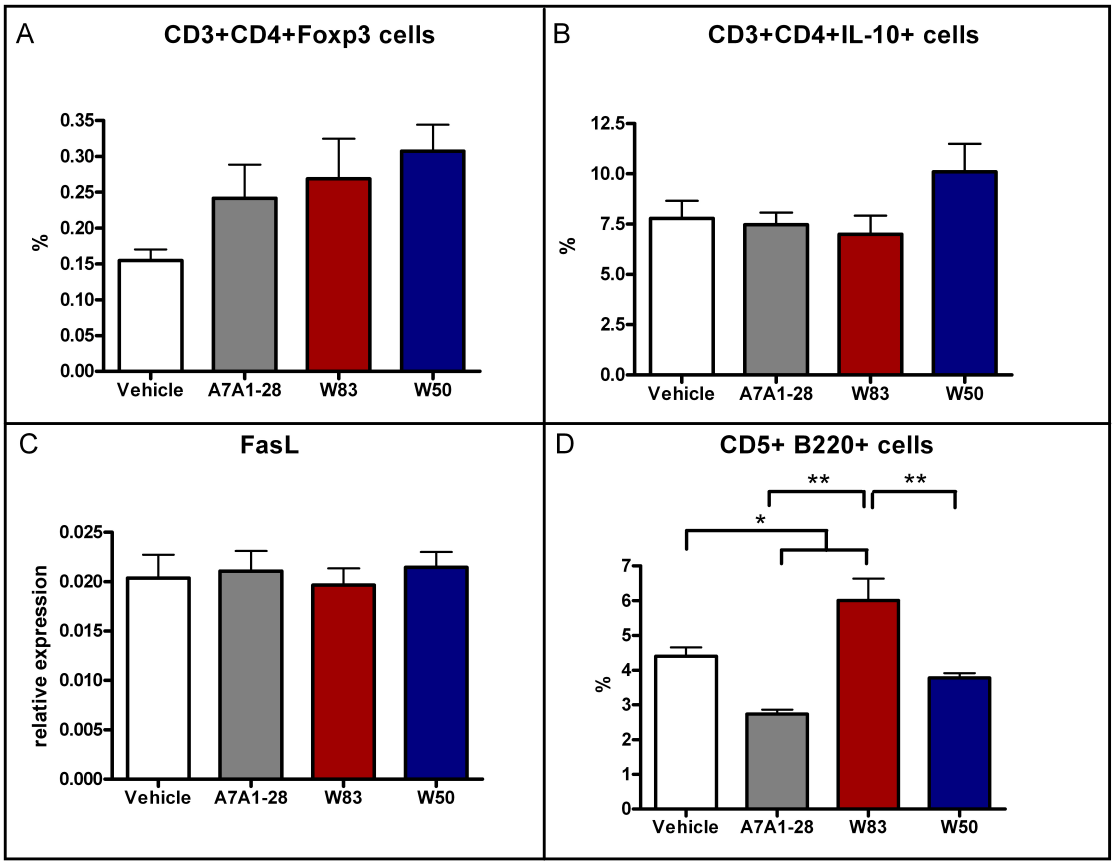

Figure 2.5. Flow cytometric analysis of splenocytes derived from mice at 42d after gavage with *P. gingivalis* for regulatory markers. (A) $CD3^+CD4^+Foxp3^+$ cell percentage, **(B)** $CD3^+CD4^+IL\text{-}10^+$ cell percentage, **(C)** mRNA FasL relative expression of non-activated splenocytes (towards GAPDH), and **(D)** $CD5^+B220^+$ cell percentage. Error bars indicate standard error. Asterisks denote differences found among groups by Tukey test (*p<0.05, **p<0.001).

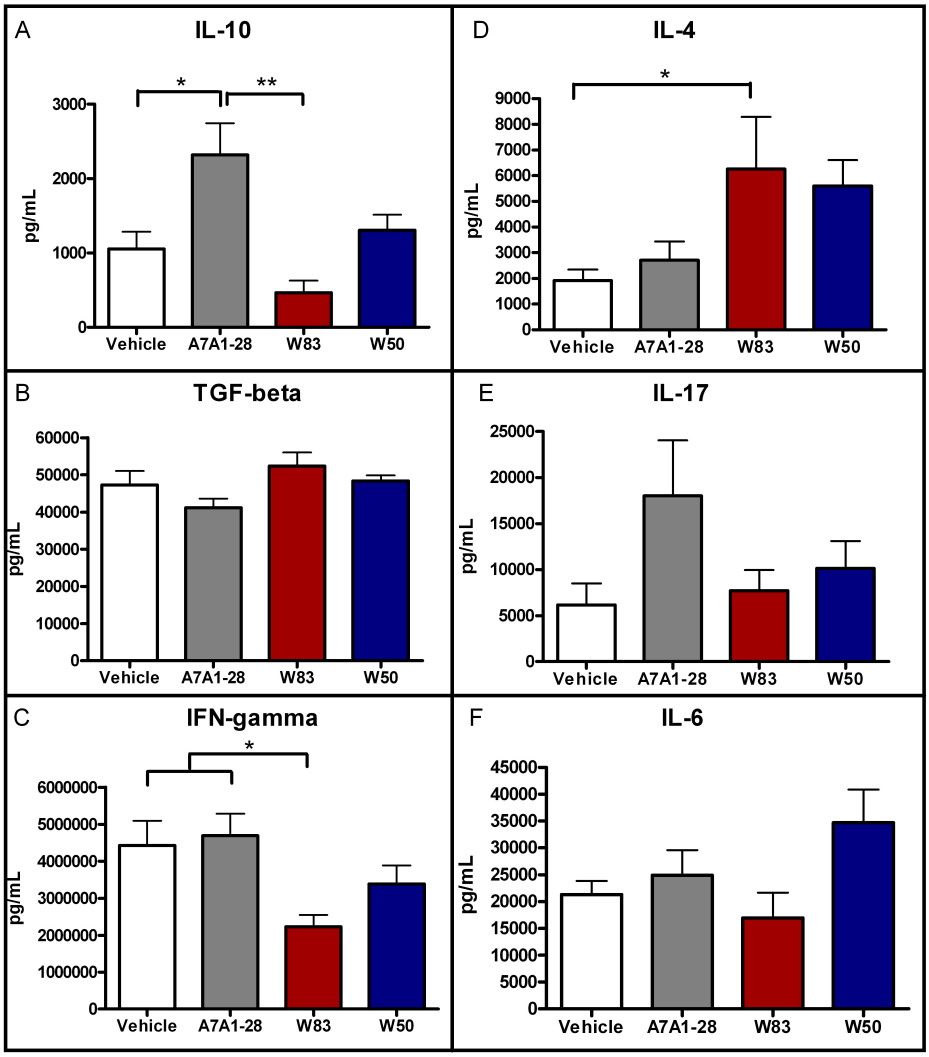

Figure 2.6. Cytokine expression of 42d post-gavage murine splenocytes treated with PMA/Ionomycin for 48h *in vitro*; (A) Differences were found among groups for the expression of IL-10 ($p=0.0006$) and no differences for **(B)** TGF-β; **(C)** IFN-γ expression was higher in A7A1-28 strain, and **(D)** IL-4 expression was higher in W83 strain. No differences were found in expression of **(E)** IL-17 or **(F)** IL-6. Experiments were performed in duplicates. Error bars indicate standard error. Asterisks denote differences found among groups by Tukey test (*$p<0.05$, **$p<0.001$).

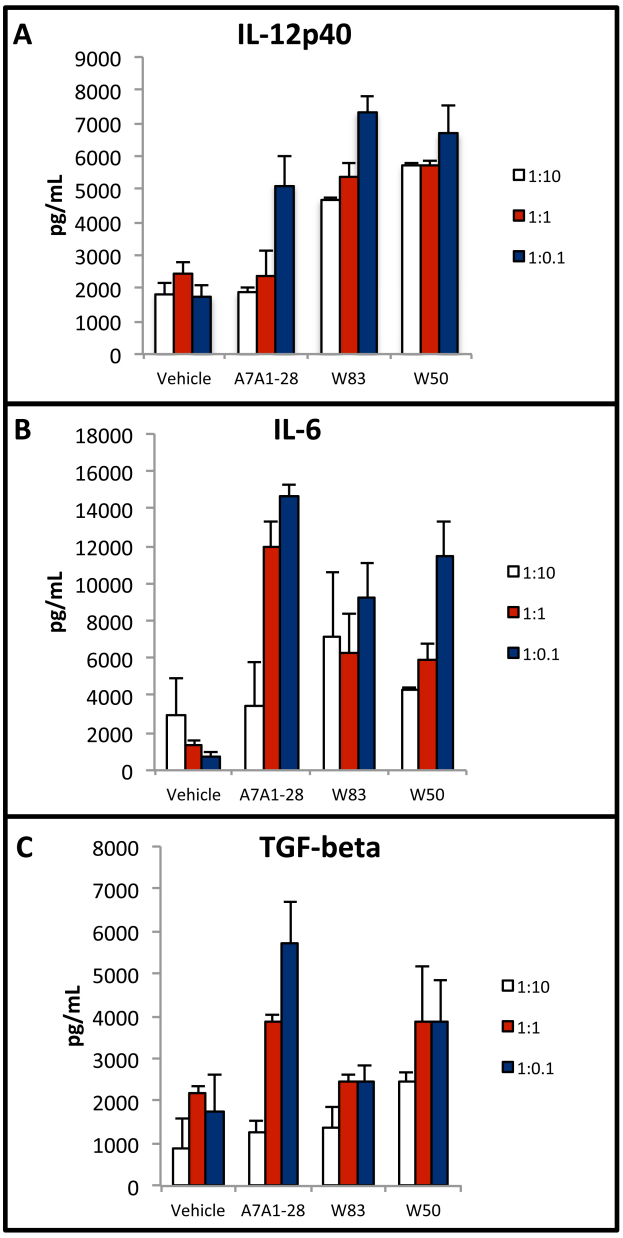

Figure 2.7. Dendritic cells stimulated with treated with *P. gingivalis* strains A7A1-28, W83, and W50 at MOI 1:10, 1:1, 1:0.1 showed an expression of **(A)** IL-12p40, **(B)** IL-6 and **(C)** TGF-β in a dose-dependent manner. Experiments were performed in duplicates. Error bars indicate standard error.

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CHAPTER III

Oral *Porphyromonas gingivalis* infection exacerbates arthritis predominantly via Th17 cell responses

ABSTRACT

Introduction: To determine the effect of chronic periodontal disease on immune activation during collagen-induced arthritis (CIA) in mice.

Materials and methods: Mice chronically infected with *Porphyromonas gingivalis* (*P. gingivalis*) in the oral cavity were induced for arthritis with either complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA). Mice were evaluated at 0, 30, 44, and 73 days post-gavage initiation. Periodontal disease establishment was evaluated by PCR of the oral microflora, alveolar bone loss by micro-computed tomography (micro-CT), and mRNA expression of Th cell responses in the gingival tissue and submandibular lymph nodes by qRT-PCR. Arthritis immune activation was evaluated by collagen-II antibody responses by ELISA, protein expression of Th cell responses of serum and collagen II-reactivated splenocytes by protein array. Spleen, inguinal lymph nodes, and paws were evaluated by qRT-PCR. Arthritis development was

assessed by visual assessment score and caliper measurements, followed by paw micro-CT analysis, histological scoring, and osteoclast numbers by TRAP-positive staining.

Results: In collagen II-immunized mice, *P. gingivalis* oral infection resulted in a trend for increased serum Th17/IFN-γ ratio and increased splenocyte numbers (splenomegaly). In the CFA group, an enhanced Th2-Th17 cytokine expression in sensitized splenocytes when re-activated with collagen II was observed. Treg cells in the inflamed paws appeared down-regulated as reflected in lower expression of Foxp3. *P. gingivalis* infection induced a greater number of osteoclasts and tissue swelling once arthritis affected the entire paw in the collagen II-CFA immunized mice. In the IFA group, an enhanced Th1-Th2-Th17 cytokine expression in re-activated splenocytes and up-regulation of paw Foxp3 expression were observed. Histologically, *P. gingivalis* increased pannus formation, bone destruction, and osteoclast numbers.

Conclusion: Together, our results indicate that chronic oral infection with *P. gingivalis* prior to arthritis induction altered Th cell-mediated responses that ultimately influenced paw swelling and bone destruction in collagen-induced arthritic mice.

INTRODUCTION

Periodontal disease is an immune-inflammatory infection of the tooth supporting structures, which include the gingival tissue, periodontal ligament, cementum, and alveolar bone. It is a major cause of tooth loss among the adult population. *Porphyromonas gingivalis (P. gingivalis)* is a Gram-negative pathogenic bacterium associated with increased risk of periodontal breakdown and disease recurrence (Socransky et al., 1998). *P. gingivalis* is shown to activate several innate immune receptors, including toll-like receptor (TLR)-2, TLR-4, nucleotide-binding oligomerization domain (NOD)-2, and protease-activated receptor (PAR)-2 which ultimately contribute to disease initiation and progression (Burns et al., 2006; Chung et al., 2010; Gaddis et al., 2011). The chronicity of periodontitis leads to extensive release of innate receptor-triggered inflammatory mediators that harm the host by increasing inflammation and bone destruction. Following activation of antigen-presenting cells, a mixed T helper (h)1/Th2 cell response takes place along with a robust induction of IgG/IgA immune responses (Verma et al., 2010). Protection against alveolar bone loss is observed in mice deficient for IL-12p40 (a major Th1-inducing cytokine) and IFN-γ when infected with *P. gingivalis* (Alayan et al., 2007; Baker et al., 1999). Clinically, IFN-γ levels are associated with progressive lesions and higher-severity of periodontitis (Honda et al., 2006) while IL-12 levels are shown to

decrease in gingival crevicular fluid following periodontal therapy (Thunell et al., 2010). High levels of IL-5 and IL-4, Th2-driven cytokines, have also been reported to be highly expressed by gingival mononuclear cells from patients with severe periodontitis (Fujihashi et al., 1993). The role of Th17 has been more recently explored. Studies show the presence of several Th17 markers in clinically diseased periodontal tissues, as well as IL-17 mediation of alveolar bone loss in murine aging-associated periodontitis (Eskan et al., 2012). While the effect of *P. gingivalis* in oral tissues is better documented, fewer studies have evaluated the systemic effect of this bacterium (Marchesan et al., 2012; Saadi-Thiers et al., 2012) and if exposure to its chronic infection may affect the development of other diseases.

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that affects 1% of the population and is associated with premature mortality, disability, and compromised quality-of-life (Brooks, 2006). Accumulating clinical evidence supports the association between periodontitis and RA (Chen et al., 2012; Mikuls et al., 2012). RA subjects show higher severity of periodontitis and number of missing teeth compared to healthy subjects (de Pablo et al., 2008). Patients with periodontitis show a prevalence of RA of 3.95% compared to 0.66% prevalence in healthy controls (Mercado et al., 2000), suggesting that the relationship between these two diseases is bi-directional. Immunity to *P. gingivalis*, but not other periodontal bacteria, is significantly associated with the presence of RA-related autoantibodies in individuals at risk for developing RA (Mikuls et al., 2012), suggesting that oral infection with certain bacteria may play

a role in the early loss of tolerance to self-antigens that occurs in the pathogenesis of RA. The direct effect of periodontal disease in RA has been clinically shown by decreased serum erythrocyte sedimentation-rate, C-reactive protein (CRP), TNF-α levels and improved disease activity score (DAS28) after periodontal treatment in patients with both RA and periodontitis (Al-Katma et al., 2007; Erciyas et al., 2012; Ortiz et al., 2009). In pre-clinical studies, mice that received oral gavage with *P. gingivalis* prior to collagen-antibody induced arthritis developed arthritis at a greater rate compared to arthritis alone, with increased inflammation and tissue destruction (Cantley et al., 2011). While systemic cytokines were not measured in this study, an increased serum CRP was observed in mice induced for both periodontitis and arthritis. Given that both RA and periodontitis are Th mediated diseases, and that cytokine modulation is utilized in clinical therapies for disease control (McInnes and Schett, 2007), increased understanding of the effect of chronic periodontitis on the immune activation stages of arthritis development is needed.

The collagen-induced arthritis (CIA) model is considered the gold-standard *in vivo* model for RA studies due to the similarities found with human RA, including pathological features and the importance of Th1/Th17 cell responses (Sarkar et al., 2009). CIA is elicited in strain-specific mice by immunization with collagen II (CII) emulsified in complete or incomplete Freund's adjuvant, which is composed of a mixture of mannide monooleate and heavy paraffin oil with or without heat-killed *Mycobacterium tuberculosis* (*M. tuberculosis*) (Brand et al., 2007). IFA is not known to contain any TLR ligand and induces a slower

development of arthritis, while CFA contains several TLR and non-TLR ligands that are shown to facilitate arthritis development (Levitz and Golenbock, 2012; Matthys et al., 1999). A significantly lower incidence and severity of collagen-induced arthritis with CFA is observed in mice deficient in TLR-4, demonstrating the importance of the innate immune response in disease development (Pierer et al., 2011). Since *P gingivalis* is known to activate cells via several innate immune receptors and lead to T cell activation, we speculated that prior exposure to its chronic oral infection would lead to an augmented immune activation induced by an adjuvant and ultimately influence arthritis development.

The present study was performed to further our understanding of the effect of *P. gingivalis* in modulating Th driven-responses and arthritis development in mice induced for collagen-induced arthritis with either CFA or IFA. Oral chronic *P. gingivalis* infection increased arthritic tissue swelling and osteoclast numbers, enhanced splenic Th2-Th17 responses, and down-regulated Treg paw response in the CFA model. When combined with the IFA regimen for induction of CIA, *P. gingivalis* chronic infection increased arthritis development and bone destruction, enhanced splenic Th1-Th2-Th17 responses, and up-regulated Treg paw response by Foxp3 expression.

MATERIALS AND METHODS

Study design. Six-week old DBA/1J male mice (Jackson Laboratory, Bar Harbor, ME) were maintained in a specific pathogen-free animal facility for biosafety level 2 infected animals. Six-week old mice were infected with *P.*

gingivalis or vehicle for 15 days and immunized for collagen-induced arthritis after an additional 15 days (at day 30) with either CFA or IFA in combination with CII, according to the experimental timeline defined in Figure 3.1. Mice were weighed and sacrificed at baseline, day 30 (D30), day 44 (D44), and day 73 (D73). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Michigan (Ann Arbor, MI) and conformed to ARRIVE guidelines for preclinical studies.

Periodontitis induction. *P. gingivalis* strain W83 (ATCC BAA-308) was grown under anaerobic conditions as previously described (Marchesan et al., 2012). Mice were given sulphamethoxazole at 0.87 mg/mL and trimethoprim at 0.17 mg/mL (Hi-Tech Pharmacal Co. Inc., Amityville, NY) in mili-Q water *ad libitum* for 10 days, followed by 3 days without antibiotics. For infection, mice were inoculated with an average $2X10^9$ CFU of bacteria in 100uL of PBS with 2% carboxymethylcellulose (CMC; Sigma-Aldrich, St Louis, MO) by oral gavage for 15 days as previously described (Kobayashi et al., 2011). The vehicle group received CMC alone. Periodontal disease was evaluated by the presence of *P gingivalis* in the oral cavity by PCR, mRNA expression in the gingival tissues and submandibular lymph nodes, and histology of alveolar bone. Alveolar bone loss was assessed by micro-computed tomography (micro-CT).

Arthritis induction. Mice were induced for arthritis as previously described (Brand et al., 2007). Briefly, chick CII at 4mg/mL in 50mM acetic acid was

emulsified in equal volumes of either IFA or CFA. IFA was composed of mannide monooleate and heavy paraffin (Fisher Scientific, Hampton, NH). CFA was composed of IFA and freshly groined *Mycobacterium tuberculosis* strain H37Ra (BD Biosciences, San Jose, CA). The emulsion of 50uL was injected intradermally at the base of the tail. No booster immunization was performed. Arthritis was scored by visual assessment score using a scale of 0-4 per limb as previously described (Sarkar et al., 2009) starting at day 44 of timeline (14 days after CIA immunizations) as follows: 0 = no swelling or redness of paws or digits, 1 = swelling or redness in 1 or 2 digits, 2 = swelling and redness over ankle or 3 or more digits, 3 = swelling and redness over ankle and midfoot or digits and midfoot, 4 = swelling and redness over entire foot or ankylosis. To further evaluate the severity of arthritis, paws that achieved a visual assessment score of 4 (or arthritis development in the entire paw) were measured in both the medial-lateral and dorsal-ventral dimensions by a blinded examiner utilizing a Lange skinfold caliper (Beta Technology Incorporated, Santa Cruz, CA) at timeline days 65, 67, 70, and 72. The most severely arthritic paw determined by caliper measurement was collected for micro-CT and histological analysis. The second most severely arthritic paw was collected for gene expression analysis.

Oral microflora analysis. For *P. gingivalis* colonization analysis, the oral microflora of mice was collected at baseline, and timeline days 16 (1 day after last gavage), 30 (2 weeks after last gavage), 37 (3 weeks after last gavage), 44 (4 weeks after last gavage), 51 (5 weeks after last gavage), 58 (6 weeks after

last gavage), 65 (7 weeks after last gavage), and 73 (8 weeks after last gavage) of the timeline (Figure 3.1). *P. gingivalis* oral infection was confirmed by PCR of Arg-gingipain (201bp).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Mice sacrificed at baseline and at days 30, 44, and 73 had the following tissues harvested: palatal gingival tissue between M1 and M3, submandibular lymph nodes, inguinal lymph nodes, and the second most severely arthritic paw determined by caliper measurement. RNA was isolated via the TRIzol (Invitrogen, Rockville, MD, USA) method and purified with RNeasy mini kit (Qiagen, Valencia, CA). mRNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). cDNA was then amplified via TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA). The following transcription factors were evaluated: T-bet, GATA-3, RORγt, and Foxp3. Gene expression was normalized with the housekeeping gene GAPDH, and relative quantification of the data generated was carried out using the comparative CT method.

Splenocyte culture and CII re-activation. After mice were sacrificed, spleens were removed, segmented, and processed for *in vitro* cultivation (Marchesan et al; 2012). Cells were plated at $5X10^6$ cells/mL, and re-activated with 100 ug/mL of highly purified lyophilized α1(II) bovine collagen obtained as described

previously (Rosloniec et al., 2010). Supernatants were collected after 5 days of culture for protein analysis.

Protein arrays. Serum collected at baseline and days 16, 30, 44 and 73 and CII-reactivated splenocytes collected at baseline and day 30, 44, and 73 were evaluated for protein expression by Quantibody Mouse TH17 array 1 (RayBiotech, Inc. Norcross, GA) according to the manufacturer's protocol for expression of the following proteins: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, IL-17F, IL-21, IL-22, IL-23, IL-28, IFN-γ, MIP-3α, TGF-β1, TNF-α. In addition, levels of anti-CII antibodies were measured in serum at days 44 and 73 in a dose-dependent manner. Briefly, 96-well plates were coated overnight with 5ug/mL chick CII, incubated with mouse serum at 1:60, 1:240, and 1:960 for 1h, followed by incubation with alkaline phosphatase-labeled goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (SouthernBiotech, Birmingham, AL) at 1:1000 for 2h, developed and read at 405 absorbance.

Micro-computed tomography (Micro-CT): Maxilla. After sacrifice, maxillae were harvested and immediately fixed in 10% formalin for 24h, following transfer to 70% alcohol. Non-demineralized maxillae were scanned at a resolution of 12x12x12 um^3 voxels using a μCT 100 cabinet cone-beam micro-CT system (Scanco USA, Inc., Wayne, PA). Analysis was performed by a calibrated masked examiner (AT) as previously described with minor modifications (Park et al., 2007). The region of interest (ROI) encompassed the coronal region of

supporting alveolar bone from the mesial edge of the cemento-enamel junction (CEJ) of M1 to the distal edge of the CEJ of M2, excluding root tissues. The average threshold gray-scale value was calculated and used as the representative value to derive the following parameters: bone volume fraction (BVF, mm^3), bone mineral content (BMC, mg), tissue mineral content (TMC, mg), and tissue mineral density (TMD, mg/cm^3) using GEHC MicroView Analysis Plus software (GE Healthcare, Little Chalfont, UK). **Paws:** After sacrifice, paws were cut above the ankle and placed in 4.5% neutral-buffered zinc-free paraformaldehyde for 8h followed by 70% ethanol as previously described (Schett and Tuerk, 2007). Analysis was performed by a calibrated masked examiner (SS) based on a previous publication (Barck et al., 2004). Briefly, the area of newly formed bone (periosteal bone), cortical bone, and total bone were measured in a cross-sectional view of the paw. The region of interest was defined in digits 2, 3, and 4 as: 1) 1.6mm from the most prominent point of the metatarsal bone in the hind paws; and 2) 1.2mm from the most prominent point of the metacarpal bone in the front paws. Bone volumes of periosteal new bone and cortical bone were discriminated based on the bone resolution of $12um^3$ and obtained using bone analysis command of GEHC MicroView Analysis Plus software (GE Healthcare, Little Chalfont, UK).

Histopathological analysis: Maxilla. After analysis by micro-CT, maxillae were decalcified in 10% EDTA (Acros, New Jersey, USA) for a period of 14d and then embedded in paraffin. Sagittal sections (4-5 μm thick) were obtained from each

maxilla at the molar region of M1, M2, and M3 and stained with hematoxylin and eosin (H&E) for descriptive analysis. **Paws:** Paws were decalcified in 14% EDTA (Acros, New Jersey, USA) for a period of 7d and then embedded in paraffin. Transverse paw sections (4-5um thickness) were stained with H&E and TRAP staining (Sigma-Aldrich, St Louis, MO). Histopathological scores of joint damage were determined in a masked fashion (JJ) for inflammatory infiltrate (inflammatory infiltrate: 0 = no inflammatory cells in the joint cavity, 1 = A few inflammatory cells in the joint cavity, 2 = Joint cavity partially or nearly completely filled with inflammatory cells with no extension outside of joint space, 3 = severe inflammation with near-total joint space filling and extension outside of space), synovitis (0 = healthy, uninflamed 1 = Synovial thickening, mild 2 = Some pannus formation and substantial thickening, 3 = extensive pannus formation with joint deformity, cartilage destruction (0 = normal, 1 = minor cartilage destruction, 2 = loss of cartilage with underlying bony destruction, 3 = extensive loss of cartilage and destructive changes), and bone involvement (0 = normal, 1 = minor surface bony erosions, 2 = bone changes limited to juxta-articular bone with some remodeling, 3 = Extensive bony changes with major remodeling and bone destruction) (Sarkar et al., 2009). Osteoclasts were identified as TRAP-positive multinucleated cells adjacent to the bone surface and counted utilizing Osteomeasure software (Osteometrics, Inc. Decatur, GA). Osteoclasts were counted in the phalanges of digits 2, 3 and 4 and expressed as bone area and bone perimeter.

Paw caliper measurement and photography. Paw thickness was blindly measured by usage of a caliper in both the medial-lateral and ventral-dorsal dimensions, as previously described (Rosloniec et al., 2010). Photographs were taken with Canon (Lake Success, NY) at a fixed focus of 12mm, in order to obtain identical distances between the mouse's paw and the camera lens.

Statistical analysis

The study consisted of a total of 8 mice/group and a total of 15 groups for the 73d analysis. The differences among groups were statistically assessed by the unpaired two-tailed Student's *t*-test (mRNA expression fold-change and arthritis development distribution), one-way ANOVA (protein expression and arthritis scoring), Dunnet's multiple comparison test (alveolar bone loss), or linear regression analysis (paw swelling). Data were analyzed by GraphPad Prism 5.0 program (GraphPad Software, La Jolla, CA), as described in the figure legends.

RESULTS

Periodontal disease establishment and development via T-bet-GATA-3-Foxp3. To determine the development of periodontal disease in mice, the oral microflora, maxillae, gingivae, and lymph nodes were collected and analyzed**.** Evaluation of the oral microflora by PCR of Arg-gingipain (201 bp) showed that mice infected only with *P. gingivalis* had the bacteria present in the oral cavity for the 73 days evaluated in this study (Table. 3.1). Mice treated with *P. gingivalis*

and CFA or IFA showed more sporadic infection, but the bacteria was detected in the majority of the mice for at least 65 days, indicating that *P. gingivalis* was present in the oral cavity of mice while arthritis was developing. PCR was negative for the presence of *P. gingivalis* at baseline prior to oral gavage, mice in the vehicle group and in mice immunized with CFA or IFA alone.

mRNA analysis of the oral-related tissues showed a predominant T-bet expression in the gingival tissues at D44, while the expression of both T-bet, GATA-3, and Foxp3 was detected in the submandibular lymph nodes at D73 in all treated groups, independent of the presence of *P. gingivalis* (Fig. 3.2). A general down-regulation of RORγt was observed at day 73 in the submandibular lymph nodes of all treated mice compared to vehicle. None of the transcription factors evaluated were detectable at D30. These results confirm the chronicity of periodontitis with an active immune response still active at 73 days after the first *P gingivalis* exposure. The increased levels of T-bet, GATA-3, and RORγt indicate the induction of a Th1-Th2-Treg response in the oral tissues either by the presence of *P. gingivalis* or by mouse systemic immunizations with CII. No additive effect was observed in mice that received both the bacteria and the induction of arthritis in the expression of the transcription factors evaluated (Fig. 3.2)

Clear signs of inflammation and tissue remodeling could be observed in histological sections of M1 and M2 regions from mice treated with *P gingivalis* at D73 (Fig. 3.3). The presence of a dense fibroblast population above the alveolar bone crest could be observed in mice gavaged with *P gingivalis* (yellow arrows)

but could not be observed in the mice gavaged with vehicle. In addition, inflammatory cells were observed in sections from mice treated with *P gingivalis* (red arrows) and an irregular alveolar bone crest could also be detected (blue arrows). These features are indicative of the presence of a moderate, chronic inflammatory event commonly observed in the gavage model for periodontitis induction. The presence of these events could not be observed in control mice.

For alveolar bone analysis by micro-CT, mice treated with *P gingivalis*, CFA or Pg-CFA showed significant bone loss with an average of 14.5% tissue mineral content loss and 12.8% bone mineral content loss when compared to vehicle (Dunnet's multiple comparison, $p<0.05$) (Fig. 3.4). Mice treated with either IFA or Pg-IFA showed a trend of bone loss, with an average of 14.6% tissue mineral content loss and 13.4% bone mineral content loss when compared to vehicle. Still, statistical differences were found between IFA and vehicle ($p<0.05$, Dunnet's multiple comparison test).

Immunization with adjuvants was not altered by the oral presence of *P gingivalis*. To determine whether the responses to CII would be influenced by *P. gingivalis* infection, levels of anti-CII antibodies were measured at D44 and D73 by ELISA. A response was observed when levels of anti-CII antibody were measured in mice treated with CFA, Pg-CFA, IFA, and Pg-IFA (Fig. 3.5). No differences were observed in the anti-CII antibody levels between days 44 and 73 in mice treated with CFA or Pg-CFA (Fig. 3.5A-B) (ANOVA $p>0.05$). The response to CII was lower at D44 in mice immunized with IFA (Fig. 3.5-C-D), but

reached the same values as mice immunized with CFA at D73 (Fig. 3.5-E-F). As anticipated, no response to CII was observed in mice treated with vehicle or *P. gingivalis* alone. This datum indicate that the B cell response to immunization was slower in IFA compared to CFA, independent of the presence of *P. gingivalis*.

Weight analysis showed that mice immunized with CFA had weight loss from day 44 to 73 of the timeline when compared to all other groups, and *P. gingivalis* oral infection did not influence the weight of mice during periodontitis or arthritis development (Fig. 3.6).

Oral gavage of *P. gingivalis* increased Th cell responses to CFA. To determine if *P. gingivalis* would affect arthritis systemic immune response, serum samples were collected at 5 different timepoints and evaluated by a protein array for 18 cytokines associated with differentiation of Th cells. As shown in Figure 3.7, IL-28, IL-17, IL-17F, and macrophage-inflammatory protein (MIP)-3α showed increased expression at D44 in both CFA and Pg-CFA treated mice (ANOVA, $p<0.05$). The serum expression of these cytokines did not appear to be influenced by the presence of *P. gingivalis* at the timepoints evaluated, either alone or in combination with CFA.

While the levels of IFN-γ were fairly uniform in arthritic and non-arthritic mice, the IL-17/IFN-γ ratios were higher on day 44 in mice induced for CFA, which is 14 days after arthritis induction (Fig. 3.8). IL-17/IFN-γ ratio is interpreted as a balance between Th1 and Th17 responses, and a higher ratio is observed in

mice that develop more severe arthritis (Sarkar et al., 2009). Interestingly, *P. gingivalis* showed a clear trend of influencing the ratio of IL17/IFN-γ in the serum of mice that were later immunized with CFA (Fig. 3.8A) when compared to mice that did not have *P. gingivalis* exposure. These results indicate the potential for *P. gingivalis* to interfere with the balance of arthritic Th immune responses. The IL17F-IFN- γ ratio was increased at day 44 in mice that were induced for arthritis via CFA, independent of the exposure to *P. gingivalis* (Fig. 3.7B).

To further characterize the ability of *P. gingivalis* exposure and oral infection to influence arthritis development, inguinal lymph nodes and the second most severely affected paw were evaluated for gene expression of the transcription factors involved in Th cell development. Expression of t-bet was significantly upregulated in the inguinal lymph nodes of mice at day 44, or 14 days after arthritis induction. No differences were found between mice exposed to CFA or Pg-CFA (Fig. 3.9A). Inguinal lymph node expression showed down-regulation of Th2 and Th17 responses of mice induced for arthritis compared to vehicle and mice exposed to *P gingivalis* alone (Fig. 3.9C,E), while the expression of Foxp3 was similar among all groups (Fig. 3.9G). This indicates that arthritic mice were actively developing a Th1 response in the lymph nodes, which did not seem to be altered by *P. gingivalis* exposure. Interestingly, the paws of mice induced for arthritis alone demonstrated a 25-fold increase in Foxp3 expression when compared to mice gavaged with *P. gingivalis* followed by arthritis induction (Fig. 3.9H). Surprisingly, mice exposed to *P. gingivalis* alone

showed a significant increase of T-bet in the paws at day 44 (Fig. 3.9B), which was not observed in arthritic mice.

To further evaluate changes in the arthritic immune response induced by *P. gingivalis oral* infection, spleens were harvested, processed for red blood cell elimination, and counted for cell number. Numbers of splenocytes significantly increased at day 30 of the timeline, or 15 days after the last gavage was performed in mice only gavaged with *P. gingivalis*, indicating that the gavage and infection was leading to a systemic response in mice (Fig. 3.10). This was also observed at day 73, indicating the chronic effect of the oral infection. Splenocyte numbers were significantly up-regulated in mice that were induced for arthritis at D73 of the timeline when compared to vehicle. The data indicates that there was an additive effect in the splenic response when mice were gavaged with *P. gingivalis* prior to arthritis induction compared to arthritis alone, showed by significantly increased splenocyte numbers at day 73 (Fig. 3.10). This data confirms that an active systemic immune response was being induced by chronic *P. gingivalis* oral infection.

Additionally, it was noted that mice gavaged with *P. gingivalis* followed by CIA induction had a significant decrease in the local inflammatory response that normally develops in the tail of mice at the site of adjuvant injection. Pictures were taken at 21 days after immunization, known as a peak of the 1-2 week period at which it occurs (Fig. 3.11). Together, the data indicated that the chronic infection with *P. gingivalis* primed the immune system of mice and altered the

systemic response for arthritis development, even with a 15-day period in between disease inductions.

Supernatants from splenocytes treated with highly purified lyophilized α1(II) bovine collagen for 5 days were evaluated for protein expression by protein array. Reactivated splenocytes showed significant increased levels of IL-17 and IL-17F at days 44 (14 days after arthritis induction) and 73 (43 days after arthritis induction) compared to non-activated splenocytes (Fig. 3.12). No differences were observed between non-activated and re-activated splenocytes in mice exposed to *P. gingivalis* only or vehicle, and no differences were observed between re-activated splenocytes from CFA and Pg-CFA groups. These data show that expression of two Th17 cytokines were directly related to CII immunization and the expression was not altered by *P. gingivalis* infection.

Interestingly, other cytokines related to a Th1, Th2, and Th17 responses were found to be differentially expressed in mice that received *P. gingivalis* followed by CFA immunization compared to CFA immunization alone. IL-28 was the only cytokine that was significantly increased after CII reactivation in CFA alone and not expressed in the Pg-CFA group, specifically at day 44 (14 days after arthritis induction; Fig. 3.13A). Th2-related cytokines IL-5 and IL-13 and Th17 related cytokine IL-22 were significantly up-regulated in mice from Pg-CFA group compared to CFA alone (Fig. 3.13B,C,F) at D73, or 43 days after CIA immunization. Significantly increased expression of IL-1β and IL-6 was found in mice from Pg-CFA group independent of CII re-activation (Fig. 3.13D,E). The data suggests that both the innate and acquired systemic response were

activated by *P. gingivalis* exposure, and the priming of cells differentially influenced the network that commonly would develop once arthritis was induced.

Arthritis development was not influenced by prior *P. gingivalis* oral infection. Arthritis development was evaluated by visual assessment score by 2 calibrated examiners (JTM and EAG) starting at day 44 of timeline (14 days after CIA immunizations). Prior infection with *P. gingivalis* did not influence arthritis progression (Fig. 3.14A-B), with no significant differences in the final mean visual assessment score or final number of paws affected by arthritis between CFA group and Pg-CFA groups (Fig. 3.14C-D) at day 72. None of the mice in the vehicle group or Pg group developed arthritis (Fig. 3.14D).

The distribution of arthritis severity based on the visual assessment scoring among paws was also statistically similar between CFA and Pg-CFA groups, in both front and hind paws (Fig. 3.15). Both bone destruction and/or periosteal new bone could be observed in paws that developed arthritis (Fig. 3.16).

To further characterize the effects of *P. gingivalis* oral gavage in arthritis development, the most severely arthritic paw determined by caliper measurement was collected and evaluated for bone loss by micro-CT and histology for arthritis severity and osteoclast quantification. Micro-CT analysis showed that arthritis development led to increased periosteal new bone in mice that were induced for arthritis with CFA and mice that received *P. gingivalis* and CFA treatments when compared to vehicle and Pg groups, with no differences

between CFA and Pg-CFA groups (Fig. 3.17E). While cortical bone destruction was present in mice that developed arthritis, it was not sufficient to be detected by the micro-CT method at 73 days (Fig. 3.17F). When mice from group CFA were compared to Pg-CFA by histologic scoring of inflammatory infiltrate, synovitis, cartilage and bone involvement both groups showed high scores for all parameters (Fig. 3.18). Osteoclast numbers were also similar between groups at day 73 of the timeline (Fig. 3.19).

Oral *P. gingivalis* infection increased arthritis severity once entire paw was affected. To more carefully investigate arthritis development, paws that achieved a visual assessment score of 4 (arthritis affecting the entire paw) were measured in the medial-lateral and dorsal-ventral dimensions utilizing a caliper on timeline days 65, 67, 70, and 72 (corresponding to 35, 37, 40, and 42 after arthritis immunization). A visual assessment score of 4 indicates that arthritis developed in the entire paw while the caliper measurement shows differences that may appear after arthritis has established in the entire paw. The visual assessment scoring system was compared to the caliper measurement in both medial-lateral and distal-lateral dimensions, which was performed in a masked fashion. The results show that when mice displayed no arthritis in the hind paw (score 0), there were no differences over time in the amount of swelling measured by caliper, either in the medial-lateral or distal-ventral dimensions, between mice from CFA and Pg-CFA groups (Fig 3.20A). However, once the mice developed arthritis in the entire paw (score 4) there were significant differences in the

amount of swelling measured by caliper in both medial-lateral and distal-ventral dimensions (Fig. 3.20B). These differences could also be observed clinically (Fig. 3.20C-E). The same clinical trend in the amount of swelling was also observed in the front paws of mice that had a visual assessment score of 4 (Fig. 3.21). However, it did not reach statistical significance between groups (data not shown).

Paws with visual assessment scores of 4 were further analyzed for the presence of osteoclasts, and the data shows that mice with higher paw caliper swelling from Pg-CFA group had increased number of osteoclasts/bone area when compared to CFA alone (Fig. 3.22A). When evaluated for periosteal new bone or cortical bone destruction, no differences could be observed between CFA and Pg-CFA groups (22.B.C). This result shows that *P. gingivalis* oral infection prior to arthritis induction by CFA influenced the amount of osteoclasts after arthritis had established. The data suggests that a distant infection may modify the systemic response of mice and change the course of arthritis development.

Oral gavage of *P. gingivalis* increased Th cell responses to IFA in mice. Serum analysis by protein array demonstrated higher cytokine expression of mice gavaged with *P. gingivalis* and immunized with IFA at day 44 compared to all the other groups (Fig. 3.23). A trend towards a higher IL-17/IFN-γ ratio was observed in the serum of mice in the Pg-IFA group compared to IFA alone.

Splenocyte analysis showed that there was a general decrease in the number of splenocytes in mice immunized with IFA at day 44, but *P. gingivalis* altered this response as seen by an increase in the numbers of cells for mice that received *P. gingivalis* prior to IFA immunization (Fig. 3.24A). Interestingly, the expression of splenic transcription factors at 44 days (14 days after arthritis immunization) was up-regulated to 15-fold for T-bet, GATA-3, and Foxp3 when compared to vehicle. An up-regulation of 5-10-fold higher was observed when IFA alone was compared to vehicle (Fig. 3.24B-D), showing that *P. gingivalis* increased activation of T-cell responses in the spleen. This expression was not observed in mice at 73 days, but rather a down-regulation of Th17 response (Fig. 3.24E).

Paws and inguinal lymph node gene expression showed minor changes of down-regulation for Foxp3 and T-bet at days 44 and 73 of the timeline (Fig. 3.25). However, a 15-fold up-regulation of Foxp3 was observed in paws from mice that were gavaged with *P. gingivalis* and induced for arthritis with IFA when compared to all other groups (Fig. 3.25).

CII-reactivated splenocytes derived from mice in the Pg-IFA group after 5 days in culture showed significant increased levels of IL-12p70, IL-5, TNF-α, IL-1β, TGF-β, and IL-23 at day 44, indicating that *P. gingivalis* oral infection increased the systemic Th1-Th2-Th17 responses in mice immunized with IFA (Fig. 3.26). At day 73, levels of TNF-α and TGF-β were elevated in both IFA groups independent of *P. gingivalis* infection.

Increased arthritis development and bone involvement induced by *P. gingivalis* in mice immunized with IFA. An earlier arthritis development (starting at timeline-day 57 or 27 days after arthritis immunizations) was noted in mice from the Pg-IFA groups compared to IFA alone (starting at timeline-day 65 or 35 days after arthritis immunizations) (Fig. 3.27A). The final mean visual assessment score and mean number of arthritic paws/group was significantly higher at timeline-day 72 (42 days after arthritis induction) (Fig. 3.27B-C). These results show that an early infection with *P. gingivalis* had the capability to influence the development and progression of arthritis when no bacterial component was present in the adjuvant at the time of arthritis induction.

The distribution of arthritis severity based on the visual assessment scoring among paws was higher in Pg-IFA groups compared to IFA alone, in both front and hind paws (Fig. 3.28).

Micro-CT analysis of the most severely affected front paw showed a trend of higher bone involvement in mice that had prior infection with *P. gingivalis* followed by IFA immunizations (Fig. 3.29). Histological scoring of the paws showed that mice gavaged with *P. gingivalis* and immunized with IFA had increased synovial thickening and pannus formation when compared to IFA alone (Fig. 30D). A non-significant trend towards an increase in the amount of inflammatory infiltrate and cartilage involvement was observed in mice that were gavaged with *P. gingivalis* prior to IFA immunization (Fig. 3.30A-C). While bone in the IFA group displayed minor erosions, Pg-IFA mice showed bone changes involving joints with remodeling being observed (Fig. 3.31D). Number of

osteoclasts in bone surfaces were also higher (Fig. 31C-D), demonstrating that *P. gingivalis* infection prior to IFA immunization had the capacity to increase the amount of bone destruction by osteoclast recruitment.

DISCUSSION

In this study, we show for the first time that a pre-existing chronic oral infection with oral bacteria *P. gingivalis* increased paw swelling and osteoclast numbers in the CFA model of arthritis induction. In addition, *P. gingivalis* increased the arthritis development, pannus formation, and bone loss in the IFA model of arthritis induction. In both models, *P. gingivalis* altered the systemic Th1-Th2-Th17 cell response and paw Treg gene expression response that normally develops against CII in the collagen-induced arthritis model. Our findings indicate that a pre-existing oral bacterial infection can induce changes in the systemic cytokine environment that ultimately influence the development of arthritis.

There is increasing evidence showing that periodontitis treatment can alter systemic markers of arthritis and improve clinical assessment of disease (Al-Katma et al., 2007; Erciyas et al., 2012; Ortiz et al., 2009). Cytokine modulation by drugs is well known to alter arthritis development in rodent models and has importantly led to the translation into clinical therapies, such as the anti-TNF treatment (McInnes and Schett, 2007). Therefore, understanding how prior

chronic periodontitis leads to changes in the cytokine network driving arthritic immune responses before clinical bone destruction takes place is of great interest for developing preventive periodontal therapies in susceptible populations.

The presence of *M. tuberculosis* in the adjuvant at the time of immunization is known to facilitate arthritis development in the CIA model of disease. Our data confirm the well-known lower incidence, delayed arthritis development, and decreased severity in mice immunized with IFA compared to CFA (Billiau and Matthys, 2001; Matthys et al., 1999), with 2/8 mice developing arthritis in the IFA model and 8/8 mice in the CFA model (Fig. 3.14 and 3.27). The addition of *P. gingivalis* led to 5/8 mice developing arthritis with a higher degree of bone destruction in the IFA model and 7/8 mice developing arthritis with increased paw swelling in the CFA group. It has been previously shown that prior *P. gingivalis* oral infection led to arthritis developing at a faster rate, with increased bone loss in the collagen-antibody induced arthritis model when compared to mice that had no prior exposure to *P. gingivalis* (Cantley et al., 2011). In their study, the arthritis model was modified to the use of a lower LPS dosage in order to achieve a less florid degree of arthritis development and allow assessment of co-morbidity. This finding is similar to the clinical outcome observed in our study, in which the CFA model that contains heat-killed *M. tuberculosis* as part of arthritis induction showed a lower effect of *P. gingivalis* in arthritis development compared to the IFA model. However, without changing the concentration of *M. tuberculosis* from the original protocol for CFA induction, we

could still observe increased clinical swelling and increased osteoclast numbers once the arthritis had developed in the entire paw in mice that received *P. gingivalis* followed by standard CFA immunization compared to CFA alone (Fig. 3.20 and 3.22). We did not find an increased progression with the addition of *P. gingivalis* in the CFA model, but could observe a slight increase in initiation in the IFA groups (Fig. 3.27). Still, both studies demonstrated that a prior chronic infection with *P. gingivalis* altered the course of arthritis development independent of the model of arthritis induction. *P. gingivalis* infection initiation after arthritis induction was shown to have no effects on arthritis development in the pristine model of arthritis (Trombone et al., 2010). Taken together, these results indicate that the timing of infection during the course of arthritis development and immune activation seems to be an important factor when evaluating the impact of an oral infection as a co-morbidity factor.

The antibody serum response to CII was not altered by *P. gingivalis* infection (Fig. 3.5). In addition, *P. gingivalis* infection alone did not alter the serum cytokine expression at the timepoints evaluated, which is in accordance to the chronic nature of the oral gavage model of disease. All mice that received CFA immunization showed a general peak of cytokine response at timeline D44 for cytokines involved in Th1, Th2, and Th17 responses (14 after arthritis induction) (Fig. 3.7). In the IFA model of disease, no dramatic changes were observed in serum cytokine expression other than IL-28 when combined with *P. gingivalis* infection (Fig. 3.23), still at very low levels. IL-28 is a type III IFN or IFN-λ conventionally regarded as a mediator of antiviral immunity that has been

recently shown to be involved in bacterial infections. IL-28 has been suggested as a potential target for RA treatment (Kotenko, 2011) but, to our knowledge, has not yet been investigated in arthritis. Rather than cytokine analysis alone, the balance between serum Th1 and Th17 cell responses were shown to be important indicators for arthritis development (Sarkar et al., 2009). In accordance with our study findings, we showed that arthritic mice had higher serum Th17/Th1 ratios for both IL-17/IFN-γ and IL-17F/IFN-γ compared to non-arthritic mice in the CFA (Fig. 3.8A-B). Interestingly, these values had a trend of being higher in mice that were previously infected with *P. gingivalis*. A trend for increase was also observed in mice that received *P. gingivalis* in the IFA model, at both days 44 and 73 (Fig. 3.23). This datum indicate that prior *P. gingivalis* infection shows the potential to affect the Th cell balance that is important for arthritis development in mice. In addition, splenic cytokines involved in the development of all Th cells were altered in mice that received *P. gingivalis* compared to mice that were induced for arthritis but had no oral infection, with a higher number of Th17-related cytokines being expressed (Fig. 3.13 and 3.26). The splenocyte population evaluated in this study contains approximately 55% B cells, 35% T cells, and 10% of other leukocytes, including macrophages, dendritic cells and natural killer cells. Activation of this mixed cell population specifically with pure CII provides a broader understanding of the changes in the immune response development of arthritis that may not be observed with single cell isolates. In the CFA model, *P. gingivalis* oral infection increased expression of IL-5 (expressed by Th2 cells), IL-13 (mainly by Th2 cells), IL-1β (by macrophages and dendritic

cells), IL-6 (by macrophages and T17 cells), and IL-22 (by dendritic cells and Th17 cells) (Fig. 3.13). In the IFA model, mice infected with *P. gingivalis* had increased expression of IL-12p70 (expressed by macrophages), IL-5 (by Th2 cells), TNF-α at day 44 (by monocytes), IL-1β (by macrophages and dendritic cells), IL-6 (by macrophages and T cells), and IL-23 (by macrophages and dendritic cells), with a trend for increasing IL-21 expression (Fig. 3.26). Mice immunized with IFA alone also showed constitutively high expression of TNF-α and TGF-β at day 73. This finding is in accordance to the literature, since TNF-α is increased in lymphoid tissues of mice immunized with IFA alone (Mussener et al., 1995). The expression of distinct cytokines by the addition of *P. gingivalis* to either CFA or IFA at different timepoints is in accordance with the knowledge that these 2 forms of CIA have different patterns of Th cell development and arthritis progression (Billiau and Matthys, 2001). This indicates that the effect of *P. gingivalis* infection as a modifying factor in arthritis is dependent on the factors that initiate arthritis development. Despite the differences, *P. gingivalis* chronic exposure in both arthritic models appears to alter the cytokine network of innate and acquired responses that would otherwise develop.

While Th cells are actively involved in disease development, Treg cells are a distinct subset of lymphocytes responsible for limiting immune responses and avoiding autoimmunity (Sarkar and Fox, 2007). Foxp3 is a transcription factor uniquely expressed by Treg cells that is critically important for their development. While we did not find expression of Treg-related cytokines in the serum or re-activated splenocytes, the paw expression of Foxp3 was greatly altered in mice

that were infected with *P. gingivalis* prior to arthritis induction in both models of arthritis induction. In the CFA model, Foxp3 expression was significantly down-regulated at D73 (43 days after arthritis induction) in mice that received *P. gingivalis* compared to CFA alone (Fig. 3.9H). Contrastingly, mice immunized with IFA alone had no expression of Foxp3 in the paws, while mice with prior *P. gingivalis* infection and IFA immunization had increased Foxp3 expression at D73 (43 days after arthritis induction) (Fig. 3.25H). It is important to note that the paws collected in the IFA treatment had minimal arthritis development, which could be consistent with the local effect of Tregs. Of importance, several clinical studies report that Treg cells are increased in RA synovium, and that ongoing inflammation and joint damage still occurs in the presence of Tregs because these cells do not appear to function normally (Sarkar and Fox, 2007). It has been shown that TNF-α inhibits the suppressive function of natural Treg cells and TGF- β1-induced Treg cells (Valencia et al., 2006) Since arthritis developed in mice with high paw expression of Foxp3 in both CFA and Pg-IFA groups, it is possible that the target cells were resistant to Treg effects in the context of the cytokine microenvironment.

The complex interactions between monocytes/macrophages and activated T cells with osteoclasts are known to be important for the development of bone destruction in RA and CIA. While Th1 and Th2 cells show inhibitory effects on osteoclastogenesis via IFN-γ and IL-4, 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). In accordance, our results showed increased serum (Fig. 3.7) and splenic IL-17 (Fig. 3.12) expression in mice that demonstrated high numbers of osteoclasts in the CFA model independent of the presence of *P. gingivalis* (Fig. 3.19). In addition, increased swelling and osteoclast numbers were found in the Pg-CFA group compared to CFA alone once arthritis developed in the entire paw (Fig. 3.20 and 3.22). Interestingly, IL-1β and IL-6 were up-regulated in splenocytes from Pg-CFA mice independent of CII activation (Fig. 3.13). IL-17 is known to increase inflammation and increase expression of TNF-α, IL-1β, and IL-6, which further increases RANKL activity (Takayanagi, 2010). Therefore, the osteoclasts in the CFA group may be dependent on IL-17, while the addition of *P. gingivalis* may have increased osteoclast numbers in the paws by activation of additional cytokines. In mice from the Pg-IFA group, which had higher number of osteoclasts and bone destruction compared to IFA alone, a higher splenic expression of IL-1 β, TNF-α, and TGF- β at day 44 (14 days after arthritis immunization) was observed (Fig. 3.26). The activation of this cytokine environment by *P. gingivalis* may have favored the higher number of osteoclasts, since IL-1β increases RANKL activity and the combination of TNF-α and TGF- β can generate pre-osteoclasts (Matsubara et al., 2012).

Our results show that the effects of *P. gingivalis* in the CFA model were greater in the hind paws when compared to the front paws, both clinically (Fig. 3.15,19-20) and histologically (Fig. 3.22). Interestingly, mice that received *P. gingivalis* and arthritis immunization by collagen-antibody induced arthritis also

reported the hind paws more commonly affected by arthritis (Cantley et al., 2011). We speculate that this could be related to the increased predisposition of hind paws for developing arthritis compared to the front paws, since DBA1/J mice develop spontaneous arthritis in the hind paws only with aging (Braem et al., 2012). Mechanical loading is known to be an important external factor that can help regulate cartilage degradation, either being beneficial or detrimental (Sun, 2010), and the hind paws are more utilized compared to the front paws. Studies evaluating the addition of more than one co-factor in arthritis development would assist in clarifying this finding.

For this study, we utilized 6-week old male mice in accordance to the literature that supports the CIA model for arthritis development. The chronic nature of both periodontal disease and CIA permitted evaluation of the mice until almost 20 weeks of age, when mice have a mature bone (Novince et al., 2011). It is well known that gender and age can influence bone metabolism (Orwoll et al., 2001; Taneja et al., 2007). Future studies should address whether the gender and age of mice are factors that can influence the impact of chronic periodontal disease in CIA.

While the effect of arthritis induction on periodontitis development was not the focus of this study, our results show that mice gavaged with *P. gingivalis* alone had consistent infection for 73 days, while mice infected with *P. gingivalis* followed by immunization with either CFA or IFA showed more sporadic infection (Table 3.1). This leads to speculation that the microflora environment is distinct in immunized mice and does not favor *P. gingivalis* colonization to the same degree

as non-immunized mice. All groups developed an average 14% bone mineral content loss compared to vehicle control (Fig. 3.4), which is in accordance with our previous results in murine chronic periodontitis (Marchesan et al., 2012). The development of periodontitis during arthritis induction is in agreement with several murine studies, including in the CIA model (Park et al., 2011), the adjuvant arthritis model (Ramamurthy et al., 2005), the chronic-antigen induced arthritis model (Queiroz-Junior et al., 2011), and the pristine-induced arthritis model (Trombone et al., 2010). The increased levels of T--bet, GATA-3, and RORγt in our study indicate the presence of a Th1-Th2-Treg response in the oral tissues induced either by the presence of *P gingivalis* or by systemic immunizations with CII. Similarly, the pristine-induced arthritis model also showed increased expression of T-bet, GATA-3, and RORγt in the submandibular lymph nodes of mice. Periodontitis development in the CIA model was associated to increased osteoclast activity and adipocyte production, and decreased osteoblastic activity in alveolar bone cells. Their results indicate that the local alveolar cells develop an altered behavior that leads to alveolar bone loss. However, the Th cell responses and oral microflora in these mice were not evaluated. Recently, a comprehensive analysis of the subgingival microbiota of patients with new-onset and chronic RA demonstrated bacterium *Anaeroglobus geminatus* correlated with the presence of anticitrullinated protein antibodies and rheumatoid factor, while Prevotella and Leptotrichia species were the only characteristic taxa observed in patients with new-onset RA irrespective of PD status (Scher et al., 2012). This implies that the oral microflora shifts may be

distinct in the RA population. Interestingly, control of the oral microflora with antimicrobial treatment stopped the development of alveolar bone loss in both the pristine model of arthritis (Trombone et al., 2010) and the chronic-antigen induced arthritis models (Queiroz-Junior et al., 2011). However, no further characterization of the microflora was performed. Increasing the understanding of the oral microflora and potential microbiota shift that may occur with arthritis induction and development is still an open question.

In summary, our data indicates that alveolar bone loss occurs in the presence of oral *P. gingivalis* or arthritic immunization via Th1-Th2-Treg responses. Further characterization of the oral microflora shift is needed. Prior oral infection of mice with *P. gingivalis* altered the immune development of arthritis mainly via Th17 cells, and increased either the swelling or arthritis development and bone destruction depending on the model of arthritis. 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.

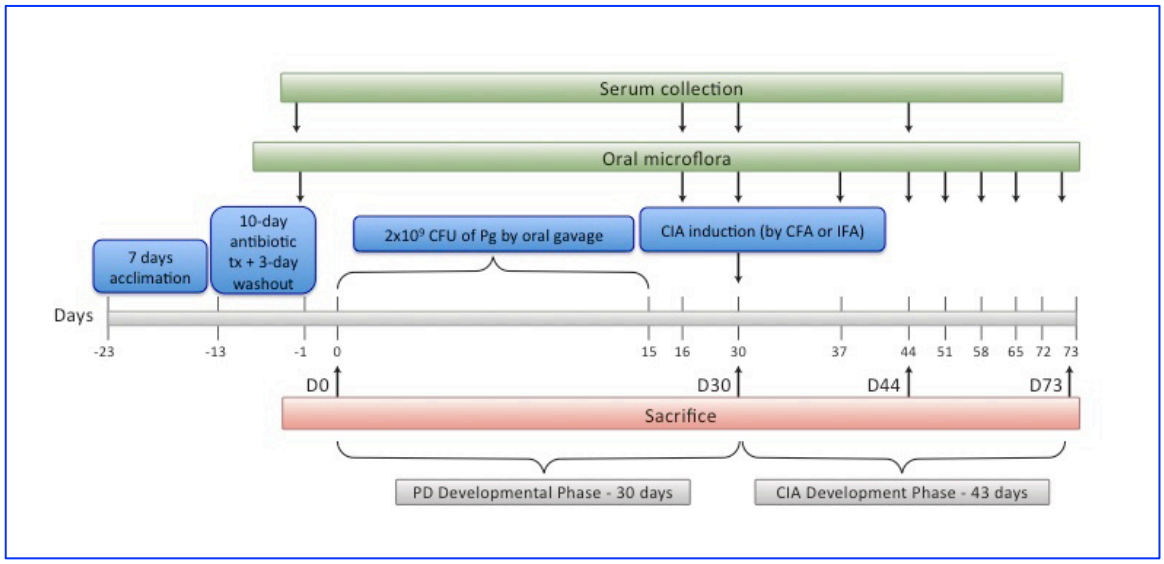

Figure 3.1. Schematic diagram illustrating the study experimental design. This timeline displays the *in vivo* experiment, including mice acclimation, antibiotic treatment, *P. gingivalis* (*Pg*) infection period, periodontitis developmental phase, collagen-induced arthritis (CIA) injection, arthritis developmental phase, oral microflora sampling, serum collection, and sacrifice.

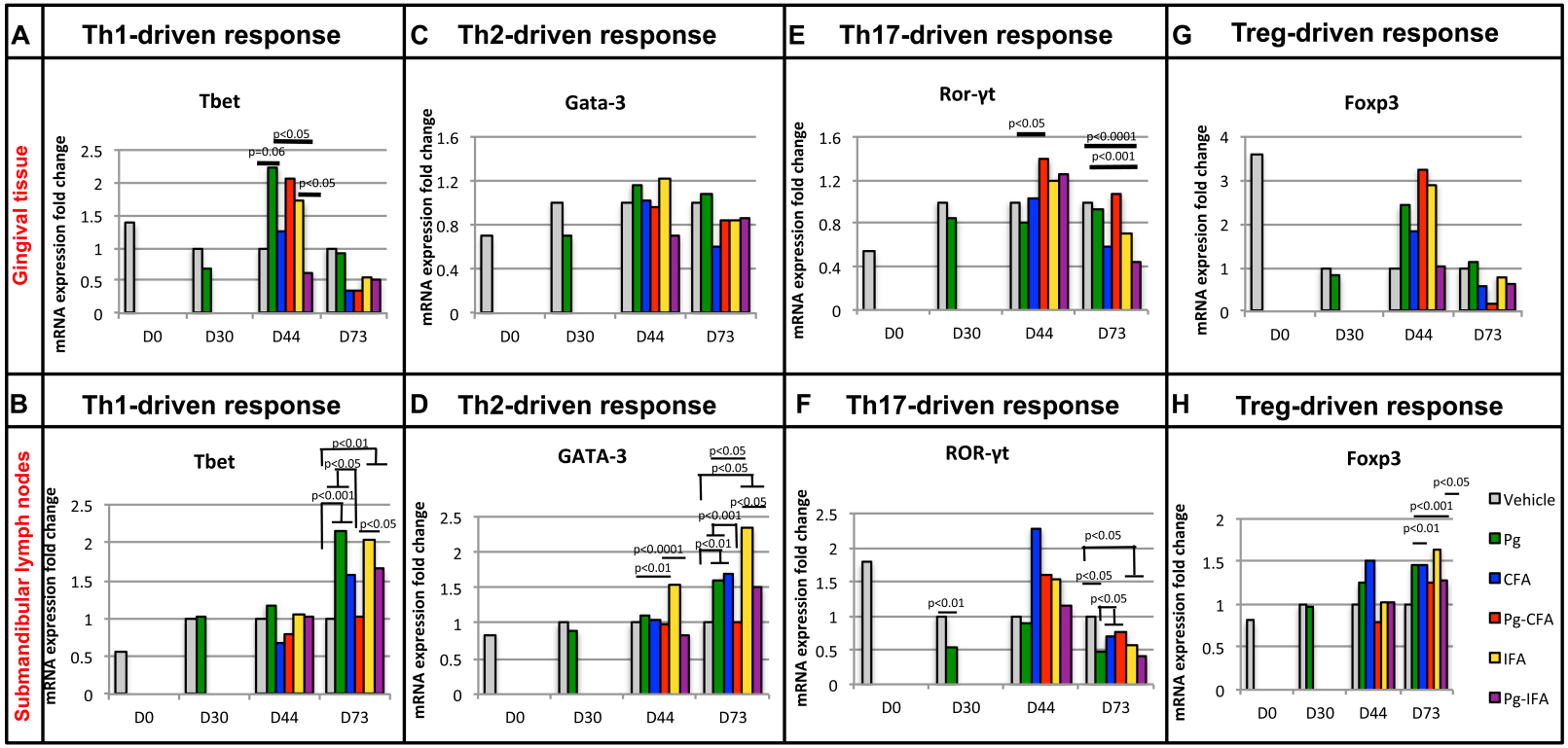

Figure 3.2. Periodontal disease development induced by *P. gingivalis* and arthritis immunization via Th1-Th2-Treg response. Transcription factors for Th cell development (A-B) T-bet, (C-D) GATA-3, (E-F) RORγt, and (G-H) Foxp3 were measured by qRT-PCR at days 0, 30, 44, and 73 of the timeline in the gingival tissues (upper rows) and submandibular lymph nodes (lower rows). Relative mRNA levels were normalized for housekeeping gene GAPDH. Data is presented as expression fold-change (n=8, p<0.05, Student's *t*-test). Group legend: Grey:Vehicle, Green:Pg, Blue:CFA, Red:Pg-CFA, Yellow:IFA, Purple:Pg-IFA.

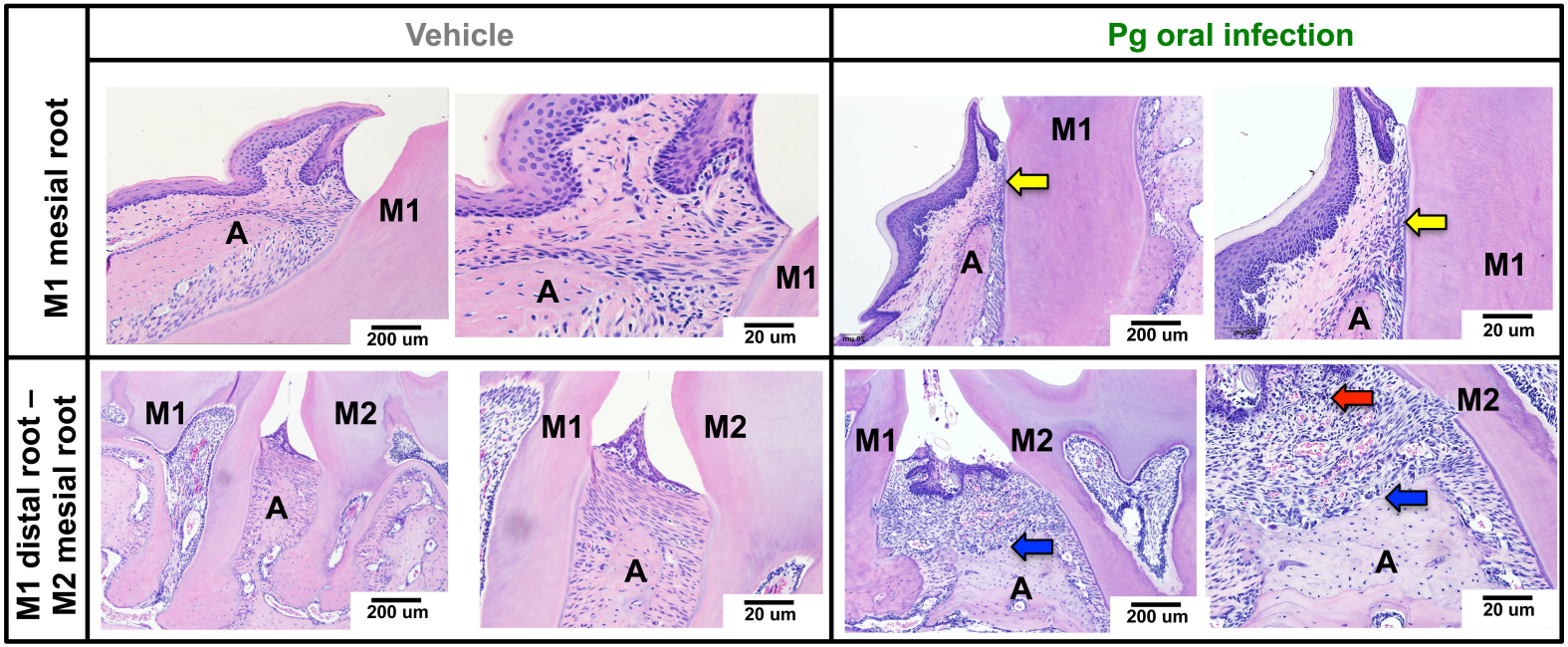

Figure 3.3. Tissue remodeling occurs by 73 days after periodontal disease initiation. Histological sagittal sections of the mesial region of M1 and the interproximal region between M1 and M2 stained with H&E. M1 and M2 = molars 1 and 2; A = alveolar bone crest; yellow arrows point to the presence of a dense fibroblast population; red arrows indicate the presence of inflammatory cells; blue arrows point to the irregular alveolar bone crest (magnifications 10X and 40X).

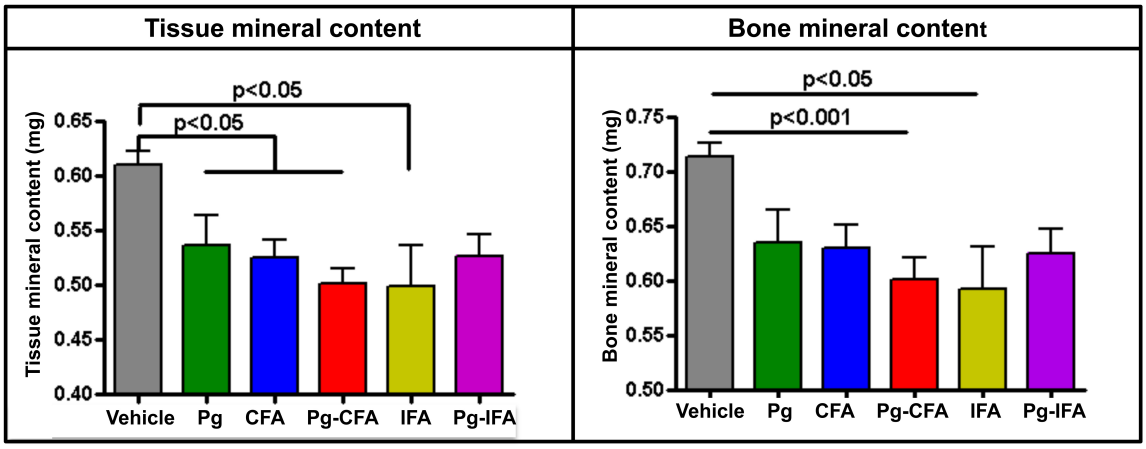

Figure 3.4. Alveolar bone loss at 73 days after periodontal disease initiation. Micro-CT analysis of the M1 and M2 region for alveolar bone loss of mice. Tissue mineral content and bone mineral content showed significant bone reduction in mice exposed to Pg, CFA or Pg-CFA when compared to vehicle at day 73. A trend was observed in mice exposed to IFA or Pg-IFA when compared to vehicle, with statistical differences for IFA and vehicle. Results are shown as a mean ±SEM (n=6-8, Dunnet's multiple comparison test, $p<0.05$).

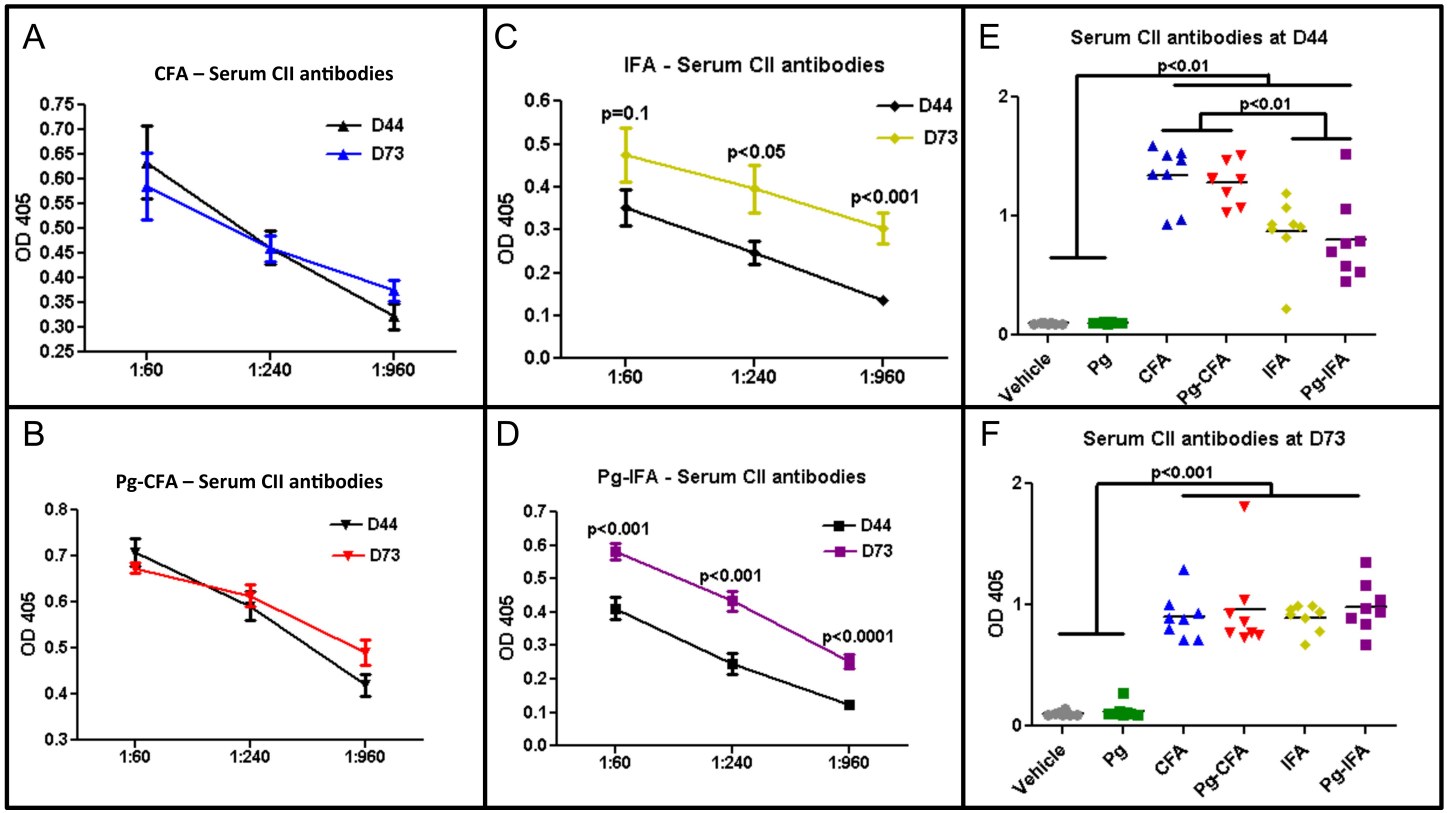

Figure 3.5. Serum anti-CII antibody response was higher in mice immunized with complete Freund's adjuvant (CFA) compared to mice immunized with incomplete Freund's adjuvant (IFA) at day 44 and not influenced by *P. gingivalis* infection. Anti-CII antibody serum response was measured by ELISA at 44 days and 73 days in mice exposed to different treatments. A dose-dependent serum concentration was used with the following dilutions: 1:60, 1:240, and 1:960. (A) mice induced for arthritis with CFA, (B) mice gavaged with *P. gingivalis* and induced for arthritis with CFA, (C) mice induced for arthritis with IFA, (D) mice gavaged with *P gingivalis* and induced for arthritis with IFA. Serum from mice exposed to all treatments was separately evaluated by timepoints, at (D) 44 days and (E) 73 days. Error bars represent ±SEM of 8 mice/group (ANOVA, $p<0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

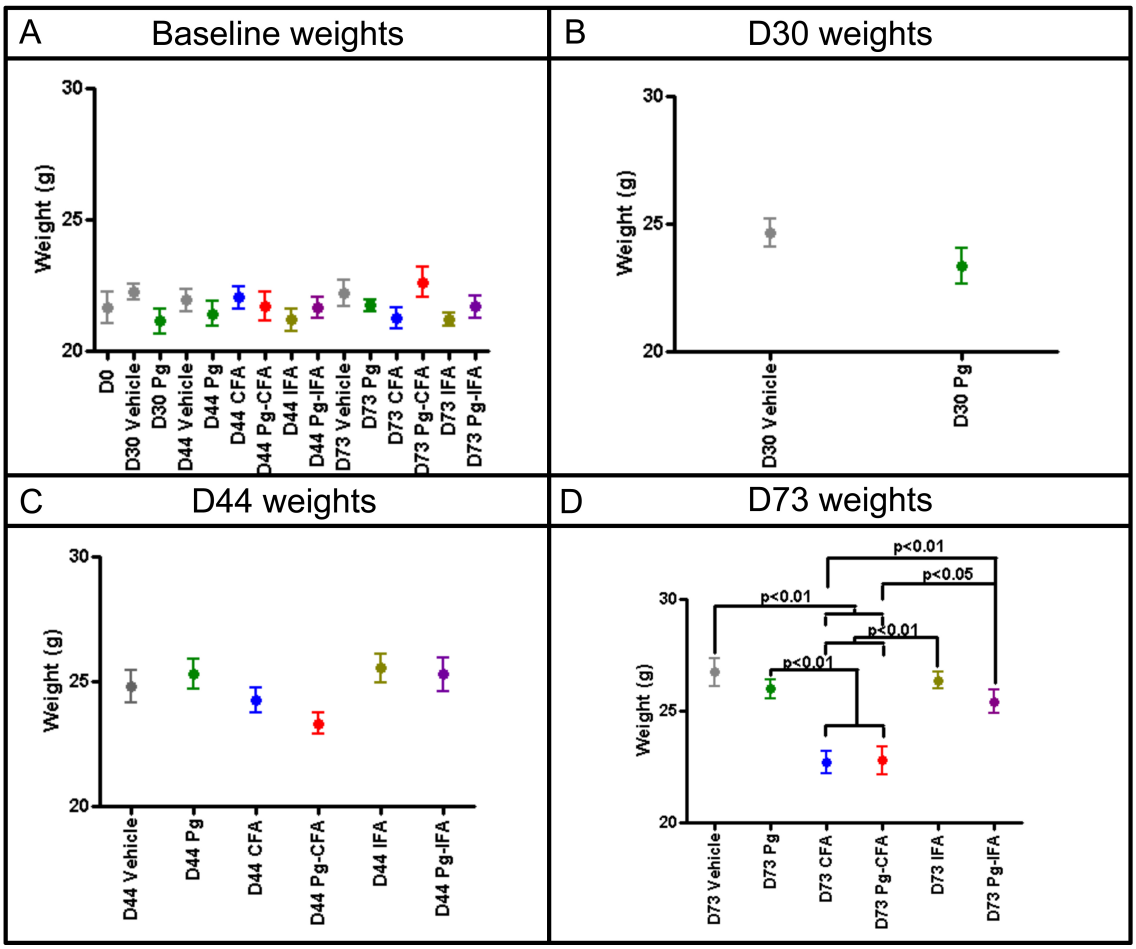

Figure 3.6. Complete Freund's adjuvant (CFA) decreased weight gain in mice independent of *P. gingivalis* infection. Mice weights were recorded at (A) baseline from all groups, at (B) D30 of timeline (15 days after the last gavage and the day that mice were immunized for arthritis) in mice from vehicle and Pg groups, at (C) D44 of timeline (14 days after arthritis induction), and at (D) D73 of timeline in mice from all groups. Error bars represent ±SEM of 8 mice/group (ANOVA, $p<0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

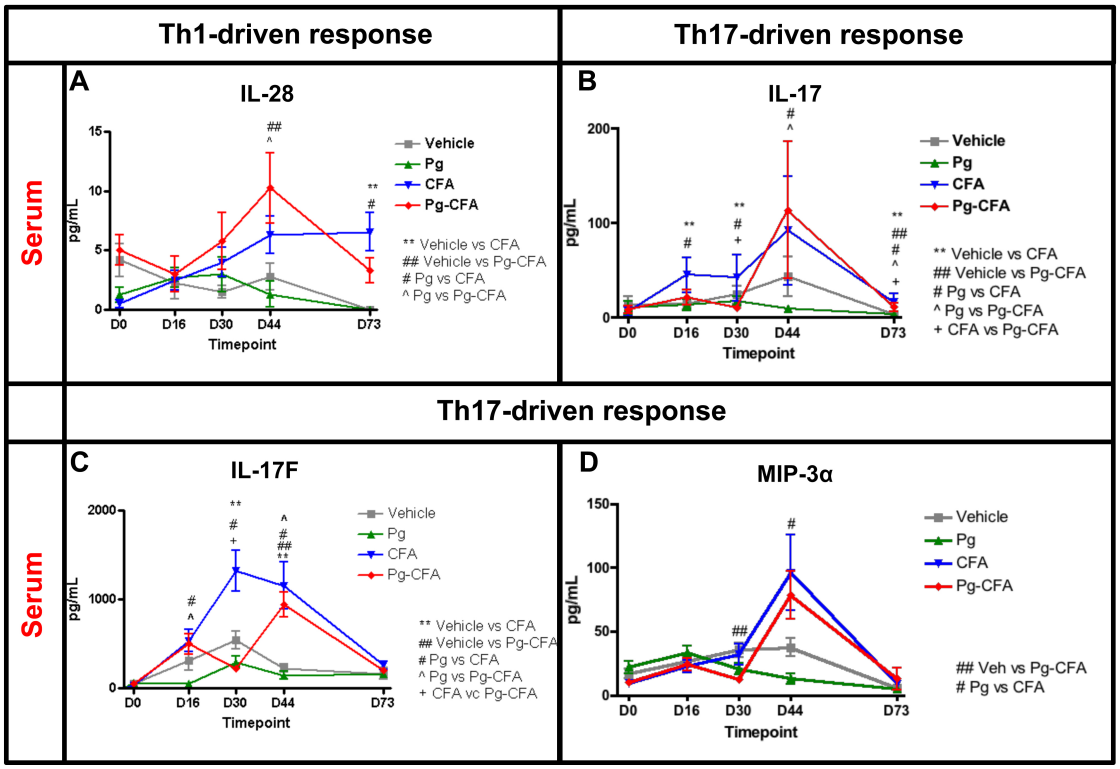

Figure 3.7. Peak of serum protein expression 14 days after arthritis immunization. Cytokine serum levels were measured by protein array at days 0, 16, 30, 44, and 73 of the timeline. Data shows expression of (A) IL-28, (B) IL-17, (C) IL-17F, and (D) MIP-3α. Error bars represent ±SEM of 8 mice/group (ANOVA, $p<0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

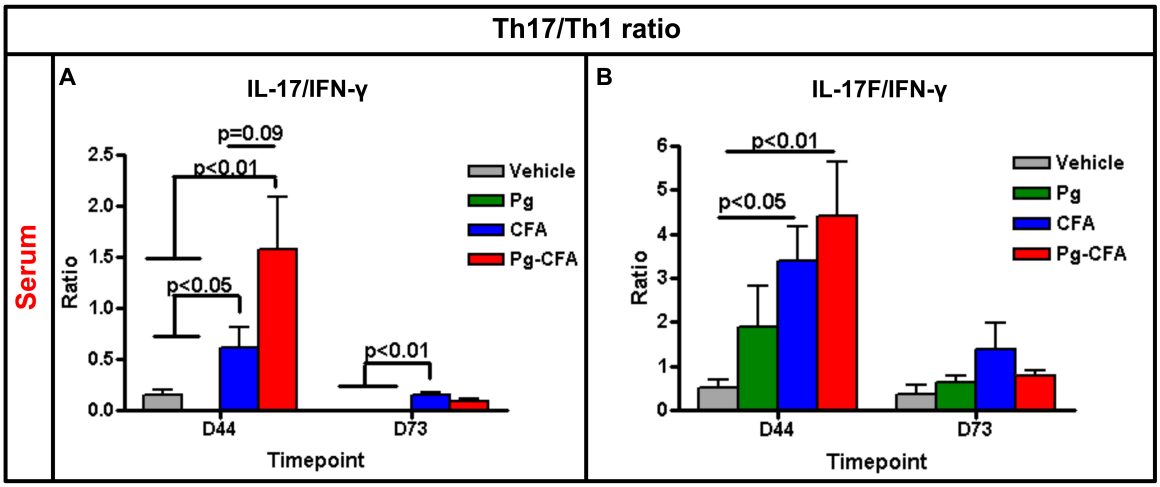

Figure 3.8. Balance of Th17/Th1 responses is higher in arthritic mice. Ratio of serum cytokines (A) IL-17/IFN-γ and (B) IL17F/IFN-γ measured by protein array at timeline days 44 (14 days after arthritis induction) and 73 (43 days after arthritis induction). Error bars represent ±SEM of 8 mice/group (ANOVA, $p<0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

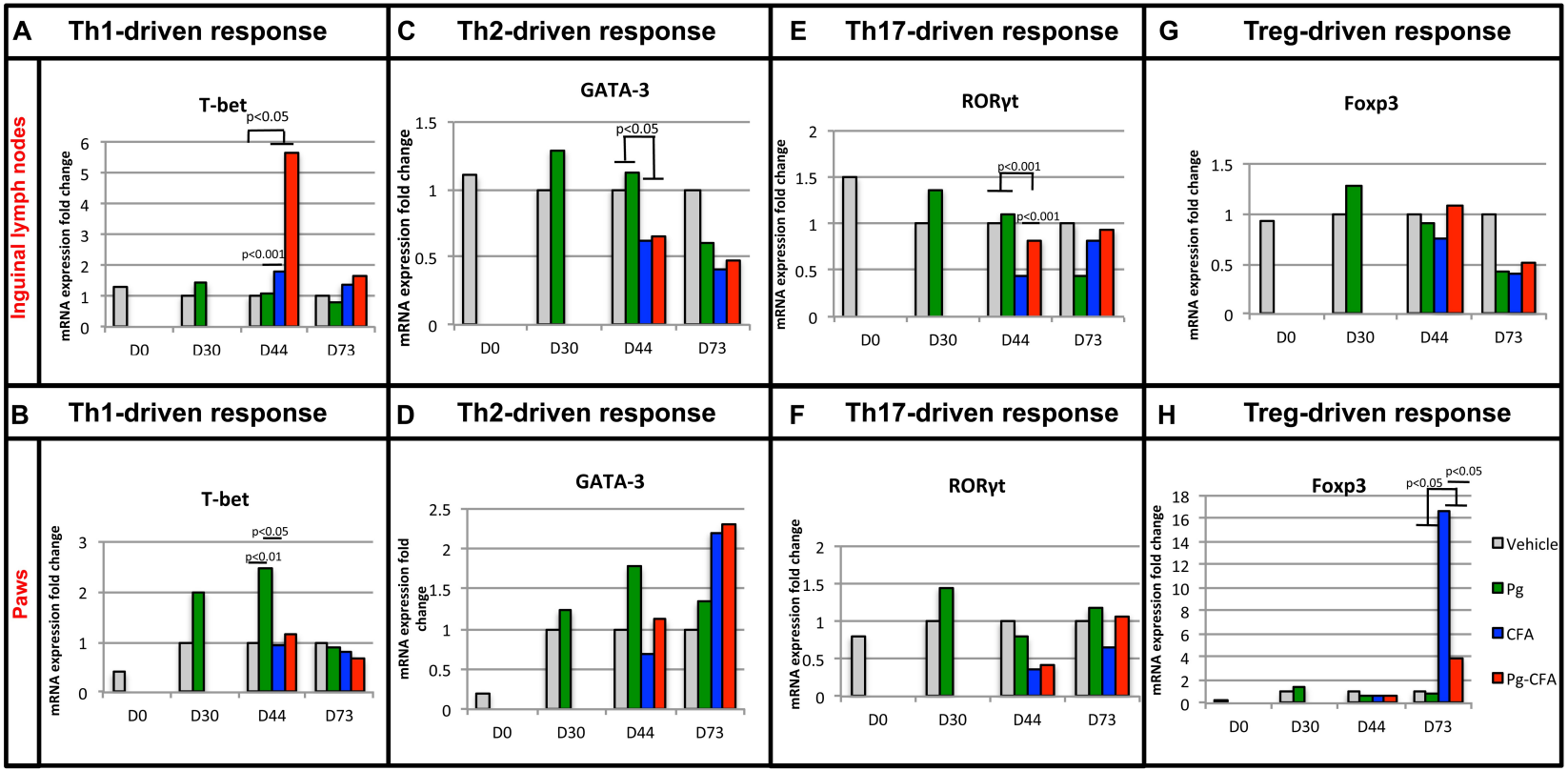

Figure 3.9. Paw Foxp3 expression is decreased by *P. gingivalis* oral infection in arthritis development with complete Freund's adjuvant. Transcription factors for Th cell development (A-B) T-bet, (C-D) Gata-3, (E-F) RORγt, and (G-H) Foxp3 were measured by qRT-PCR at days 0, 30, 44, and 73 of the timeline in the inguinal lymph nodes (upper rows) and paws (lower rows). Relative mRNA levels were normalized for housekeeping gene GAPDH. Data is presented as expression fold-change (n=8, $p<0.05$, Student's *t*-test). Group legend: Grey:Vehicle, Green:Pg, Blue: CFA, Red:Pg-CFA.

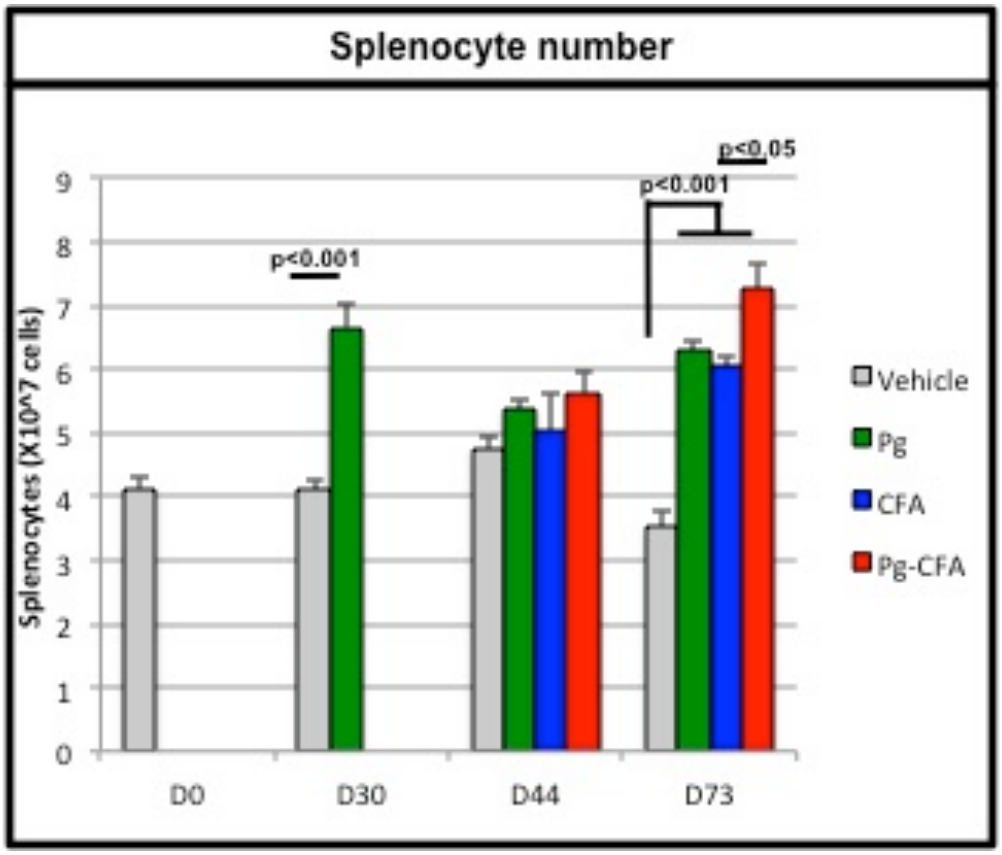

Figure 3.10. Increased systemic immune response induced by *P. gingivalis* and collagen-induced arthritis induced with complete Freund's adjuvant. Splenocytes were evaluated for cell number at days 0, 16, 44, and 73 of the timeline. Splenocyte numbers represent 8 mice/group, error bars represent ±SEM (ANOVA, $p<0.05$). Group legend: Grey:Vehicle, Green:Pg, Blue:CFA, Red:Pg-CFA.

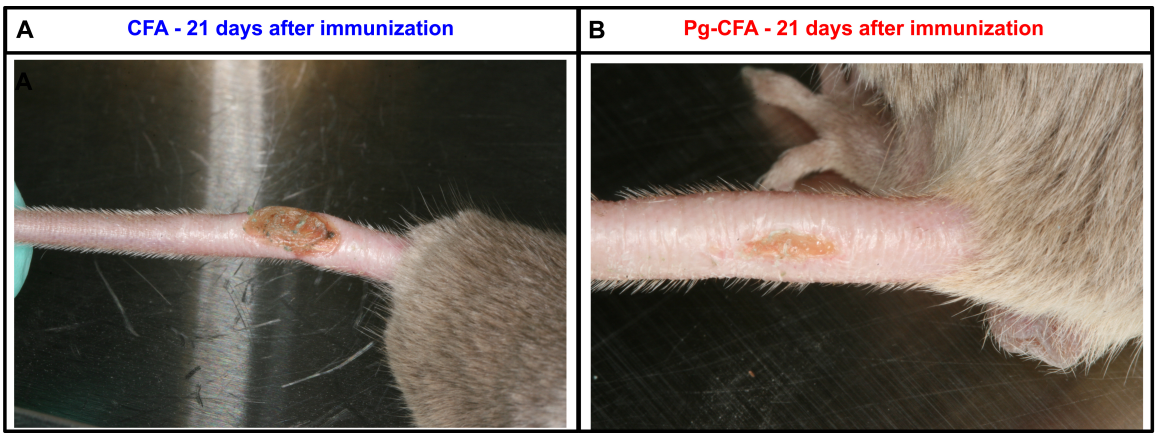

Figure 3.11. Reactive response at the site of injection for arthritis with CFA was reduced in mice previously exposed to *P. gingivalis*. (A) Representative picture illustrating normal reactive response of murine tails at 21 days after injection with 50uL chick type II collagen at 4mg/mL in 50mM acetic acid emulsified in equal volume of CFA of complete Freund's adjuvant (CFA) and (B) and mice that were gavaged with *P. gingivalis* prior to arthritis induction. CIA induction was performed consecutively in both groups with the same emulsion.

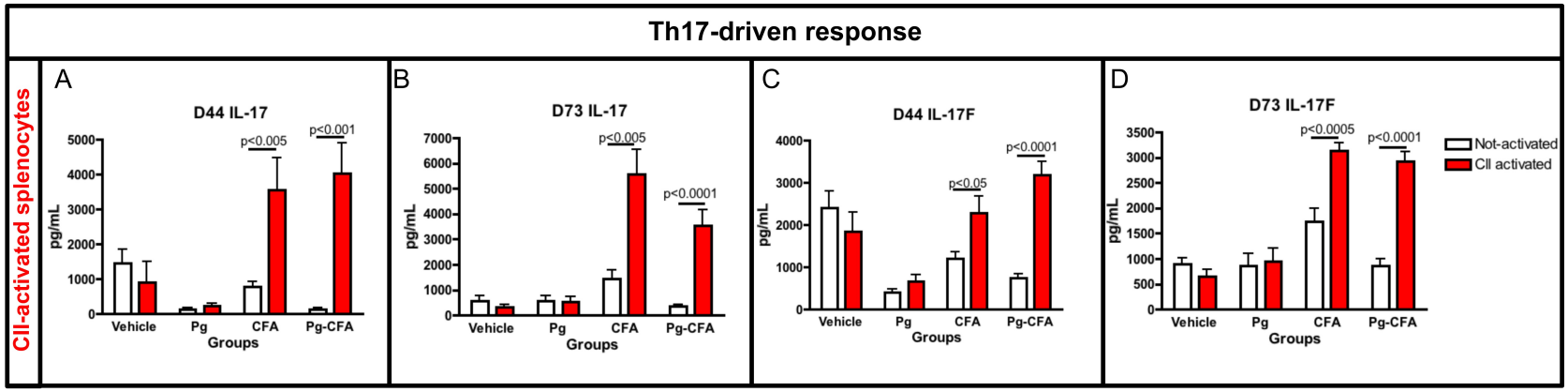

Figure 3.12. Arthritic mice showed increased expression of IL-17 after collagen II re-activation, with no influence induced by prior *P. gingivalis* infection. Murine splenocytes were isolated from mice in the vehicle group, mice that received *P. gingivalis* oral gavage, CFA immunization, or *P. gingivalis* oral gavage followed by CFA immunization and re-activated *in vitro* with 100 ug/mL of highly purified lyophilized α1(II) bovine collagen. After 5 days in culture, supernatants were collected and evaluated for (A,B) IL-17 and (C,D) IL-17F by protein array at days 44 and 73. Error bars represent ±SEM of 8 mice/group (ANOVA, $p<0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

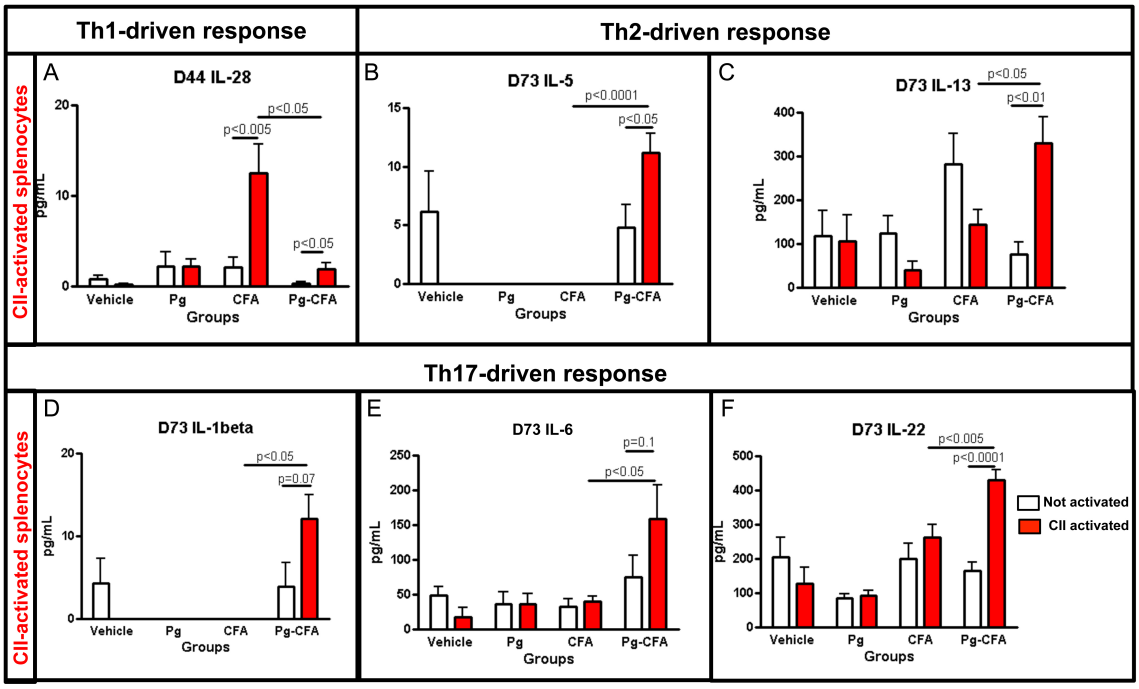

Figure 3.13. Mice exposed to *P. gingivalis* prior to complete Freund's adjuvant (CFA) immunization had increased splenic Th2-Th17 responses to CII. Murine splenocytes were isolated from mice from vehicle group and mice that received *P. gingivalis* oral gavage, CFA immunization and *P. gingivalis* oral gavage followed by CFA immunization at days 44 and 73 of the timeline. Splenocytes were re-activated *in vitro* with 100 ug/mL of highly purified lyophilized α1(II) bovine collagen for 5 days. Supernatants were collected and evaluated for (A) IL-28, (B) IL-5, (C) IL-13, (D) IL-1β, (E) IL-6, and (F) IL-22 by protein array. Error bars represent ±SEM of 8 mice/group (Student's *t*-test, $p<0.05$ and ANOVA, $p<0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

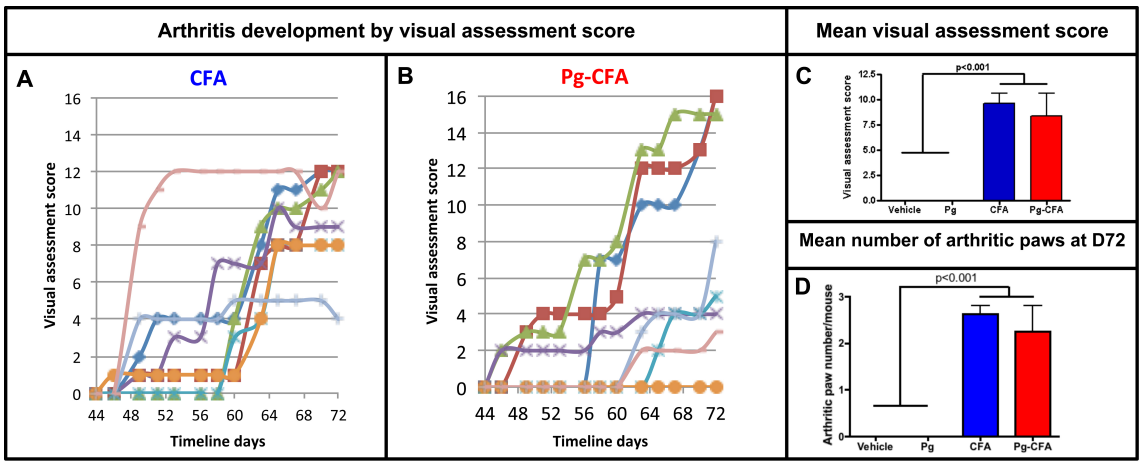

Figure 3.14. Mice exposed to *P. gingivalis* and CFA had similar arthritis progression compared to CFA alone. Arthritis development in (A) mice induced for arthritis with complete Freund's adjuvant (CFA) and (B) gavaged with *P. gingivalis* followed by CFA immunization evaluated by the visual assessment 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) number of paws that developed arthritis by the visual assessment score in vehicle, Pg, CFA, and Pg-CFA groups. Data represents ±SEM of 8 mice/group (ANOVA, $p<0.05$ followed by Tukey's multiple comparison test, indicated in the figures).

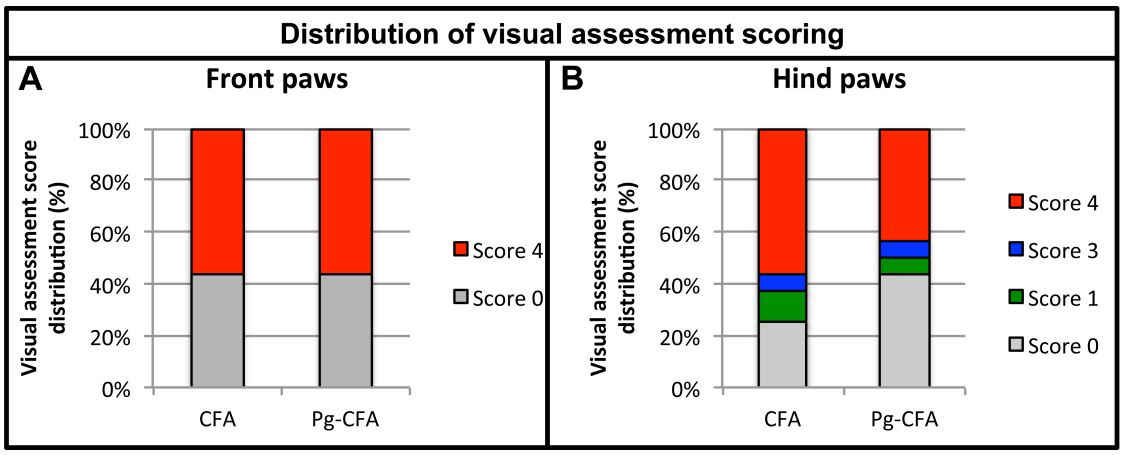

Figure 3.15. Arthritic swelling paw distribution was similar between CFA and Pg-CFA groups. Final swelling distribution at 72d of the timeline, or 42 days after arthritis induction, evaluated by visual assessment score in (A) front and (B) hind paws and represented as percentage of each score/group. Data represents 16 front or hind paws/group (Student's *t*-test, $p<0.05$).

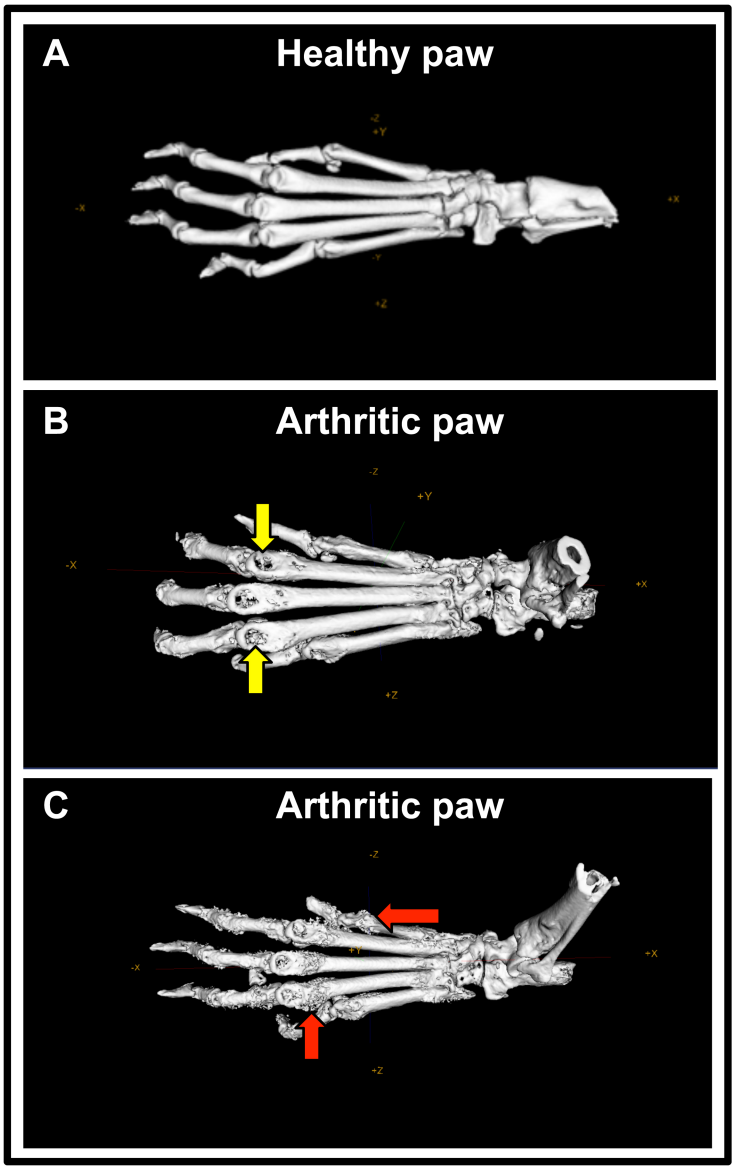

Figure 3.16. Bone resorption and periosteal new bone in paws that developed arthritis. Representative images of micro-computed tomography of paws that (A) did not develop arthritis, and paws that developed arthritis that had (B) bone resorption (pointed by yellow arrows), and (C) periosteal new bone (pointed by red arrows).