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The Role of CXCL9 and CXCL10 in Periodontal Bone Loss

A thesis submitted in partial satisfaction of the  
requirements for the degree Master of Science in  
Oral Biology

by

Elissa Beth Green

2018

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ABSTRACT OF THE THESIS:

The Role of CXCL9 and CXCL10 in Periodontal Bone Loss

by

Elissa Beth Green

Master of Science in Oral Biology

University of California, Los Angeles 2018

Professor Flavia Queiroz de Mo Pirih, Chair

**BACKGROUND:** Periodontitis is an infectious, inflammatory disease resulting in destruction of the supporting tissues of the teeth. While bacterial biofilm is central to disease pathogenesis, the host response plays an important role in the severity and progression of periodontitis. Studies have shown a strong genetic influence, accounting for as much as 50% of disease presentation. Previous work in our laboratory utilized a Genome-wide Association Study (GWAS) with a Lipopolysaccharide (LPS)-induced periodontitis murine model to identify candidate genes associated with periodontitis. Using GWAS, mRNA, and protein expression data, chemokines Cxcl9 and Cxcl10 were found to be associated and significantly upregulated in a high bone loss strain C57BL/6J compared to bone loss resistant strain A/J. Cxcl9 and Cxcl10 both exert their function through the receptor CXCR3.

**OBJECTIVE:** The purpose of this study was to investigate the influence of Cxcl9 and Cxcl10 on LPS-induced periodontal bone loss by blocking their action utilizing a CXCR3 knockout mouse.

MATERIALS AND METHODS: 12 wild-type (WT) and 12 CXCR3 knockout (KO) C57BL/6J mice were included in this study. Periodontitis was induced using *P.gingivalis*-LPS injections between the maxillary first and second molars 2x/week for 6 weeks. Following sacrifice, maxillae were scanned (microCT) and bone loss quantified. Histologic analysis of osteoclasts and pro-inflammatory mediators was performed.

RESULTS: Deleting CXCR3 demonstrated ~50% reduction in bone loss after LPS-injections compared to WT mice. 3D volumetric analysis showed no significant differences in initial bone volume/tissue volume (BV/TV) between CXCR3 KO and WT animals, indicating the changes observed were due to LPS treatment and not inherent differences in bone quality. Histologically, an increase in cellular infiltrates was seen in WT LPS treated mice compared to CXCR3 KO LPS treated mice, visualized through H&E and COX-2 immunostaining. Quantification of osteoclasts through TRAP staining revealed significantly more TRAP+ cells in WT LPS treated compared to CXCR3 KO LPS treated mice, correlating with the increased bone loss seen in the WT LPS treated animals.

CONCLUSION: CXCR3 and binding chemokines Cxcl9 and Cxcl10 are likely key players in the maintenance and amplification of inflammatory pathways. CXCR3 may be a possible target for modulating the host response in periodontitis by dampening the inflammatory cascade following LPS-stimulation. Further work is needed to characterize the CXCR3 pathway and validate other candidate genes

associated with LPS-induced bone loss. The ultimate goal is to identify patients at high risk for periodontal disease and manage them with individualized treatment.

The thesis of Elissa Green is approved.

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2018

## TABLE OF CONTENTS:

Abstract	ii
Committee	v
List of Figures	vii
Acknowledgements	viii
Introduction	1
Materials and Methods	3
Results	8
Discussion	10
Figures	14
References	20



## LIST OF FIGURES:

**Figure 1:** Hiyari et al. preliminary data

**Figure 2:** Deletion of CXCR3 *in vivo* causes a reduction in bone loss

**Figure 3:** Radiographic assessment of bone volume/tissue volume (BV/TV) in WT and CXCR3 KO mice

**Figure 4:** Histological assessment of inflammation in WT and CXCR3 KO mice

**Figure 5:** Histological assessment of osteoclast numbers in WT and CXCR3 KO mice

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## INTRODUCTION:

Periodontitis is a multifactorial, infectious disease resulting in destruction of the supporting tissues of the teeth. It is a prevalent condition and major cause of tooth loss, affecting 46% of adults in the United States over age 30 and 68% of those over the age of 65 (1). The etiology of periodontitis is bacterial biofilm formation on the root surface, but it is the exaggerated inflammatory host response that results in tissue breakdown. While any composition of bacterial plaque can result in gingivitis, the presence of specific “red complex” microbes must be present to initiate periodontitis (2,3). These pathogens, particularly the keystone pathogen *P. gingivalis*, can induce an altered composition and increased virulence of the periodontal microbiota, resulting in a dysbiotic state which can trigger inflammatory bone loss (4). However, the simple presence of these bacteria is not sufficient to cause disease. Many risk factors have been identified as contributors to disease progression, including age, genetics, systemic diseases (e.g. diabetes mellitus, HIV, cardiovascular disease), smoking and stress.

Previous work in our laboratory explored the genetic component of periodontitis utilizing a validated, reproducible murine bone loss model (5). In analyzing 104 strains of the Hybrid Mouse Diversity Panel (HMDP), a collection of inbred and recombinant inbred strains of mice bred to capture a wide genetic diversity, it was found that each mouse strain had different degrees of bone loss to the same stimulus (Fig. 1A). Of the parental strains, C57BL/6J was the most susceptible strain with fivefold higher bone loss compared to the most resistant strain, A/J

which exhibited minimal alveolar bone loss (Fig. 1B). This strain-dependent variable response to *P.g*-LPS induced periodontitis demonstrated alveolar bone loss to be a genetically modified trait (6). Utilizing a genome-wide association study (GWAS), Cxcl9 and Cxcl10 were identified as being associated with the bone loss phenotype. This was confirmed with microarray analysis where Cxcl9 induction had a 38.87-fold difference and Cxcl10 had a 19.23-fold difference in C57BL/6J compared to A/J mice, and immunohistochemistry (IHC) which revealed a significantly increased presence of Cxcl10 in C57BL/6J compared to A/J mice (7).

Cxcl9 and Cxcl10 are pro-inflammatory chemokines that are secreted from tissue resident cells including monocytes, keratinocytes, fibroblasts, and endothelial cells in response to an infectious stimulus. They act to recruit immune cells, particularly Th1 cells, cytotoxic T-cells, and natural killer (NK) cells, to the site of infection. They elicit this action by binding to chemokine receptor CXCR3, which is expressed on Th1 cells, cytotoxic T cells, and NK cells. In binding CXCR3 and triggering the chemotaxis of these immune effectors to the site of infection, they result in the release of more pro-inflammatory mediators and increased lymphocyte chemotaxis, thus amplifying the inflammatory response (8-11).

To further investigate the role of the Cxcl9 and Cxcl10 pathway in LPS-induced periodontal bone loss, our study employed a CXCR3 knockout (KO) mouse. By inducing the loss of function of Cxcl9 and Cxcl10, we would expect to see reduced periodontal bone loss and reduced presence of inflammatory cells and osteoclasts following *P.gingivalis*-LPS stimulation.

## MATERIALS AND METHODS:

### **Mice**

Seven-week-old female C57BL/6J wild-type (WT) and B6.129P2-Cxcr3<sup>tm1Dgen</sup>/J homozygous chemokine receptor CXCR3 knockout (KO) mice were bred and purchased from Jackson Laboratories (Bar Harbor, ME, USA). Our study adhered to the Chancellor's Animal Research Committee at the University of California, Los Angeles approved protocol and guidelines. Mice were housed at UCLA for the duration of the study in a temperature and light controlled environment and fed a standard chow.

### **Induction of Periodontitis**

Mice were randomly divided into treatment as follows with two-injection and twelve-injection time points:

1. 2-injection Group
  - a. 3 WT (control): no injections
  - b. 3 KO (control): no injections
  - c. 3 WT (test): *P.g*-LPS injections 2x/wk for 1 wk
  - d. 3 KO (test): *P.g*-LPS injections 2x/wk for 1 wk
2. 12-injection Group
  - a. 3 WT (control): no injections
  - b. 3 KO (control): no injections
  - c. 3 WT (test): *P.g*-LPS injections 2x/wk for 6 wks

d. 3 KO (test): *P.g*-LPS injections 2x/wk for 6 wks

Mice were anesthetized with 3% isoflurane administered through a nose cone. Utilizing a microscope (Leica Microsystems, Buffalo Grove, IL, USA), injections of 2  $\mu$ L (10mg/mL) of *P.g*-LPS (InvivoGen, San Diego, CA, USA) were placed between the first and second maxillary molars on the right and left sides of the maxilla two times a week for 1 week in the 2-injection group and 6 weeks in the 12-injection group. Injections were performed using a 10  $\mu$ L Hamilton syringe with a 33-gauge needle (Hamilton Company, Reno, NV, USA). No injections were performed in control animals as it was previously shown there were no significant differences between vehicle-injected and non-injected animals (12). No overt signs of soft tissue damage or inflammation were observed during the course of injections. Animals were sacrificed 24 hours following the final injection. Maxillae were dissected and fixed in 10% buffered formalin for 48 hours and stored in 70% ethanol for further analysis.

### **Micro-computed tomography analysis**

Maxilla were scanned using a micro-computed tomography ( $\mu$ CT) scanner (Skyscan 1172; Skyscan, Aartselaar, Belgium) with a voxel size of 10  $\mu$ m and x-ray energy of 55 KVp and 181  $\mu$ A. Each scan was conducted over a period of 21 min with steps of 0.4°. Ten frames were averaged and a 0.5  $\mu$ m aluminum filter was used. Virtual image slices were reconstructed using the cone-beam reconstruction software version 1.5 based on the Feldkamp algorithm. Volumetric data was converted to

DICOM format and imported into Dolphin software (Dolphin Imaging, Chatsworth, CA, USA) for bone loss analysis.

Utilizing Dolphin software, maxillae were oriented for the first and second molars individually with the root parallel to the sagittal plane and the cemento-enamel junction (CEJ) parallel to the transverse plane. Utilizing the sagittal plane at the interproximal contact point of the first and second molar crowns, the distance between the CEJ and the alveolar crest were measured at the distal surface of the first molar and mesial surface of the second molar just below the contact point and 0.2mm palatal to the contact point. Measurements were performed on the right and left sides. The average of the control sites was subtracted from the measurements of the LPS-injected sites to give a value representative of the net bone loss at the LPS-injected sites.

To evaluate initial bone quality between CXCR3 KO and WT mice, 3D volumetric analysis was performed in the mesial femur distal from the growth plate and in the maxillae in between the first and second molars at the injection site.

Femurs were scanned using Skyscan micro-CT (Model 1172; Kontich, Belgium) at a resolution of 12 $\mu$ m. DataViewer (V.1.5.2; Bruker, Billerica, MA) software was used to orient femurs parallel in the sagittal and coronal planes. CTAn (V.1.16; Bruker, Billerica, MA) software was used for 3D volumetric analysis in the axial plane. A region of interest (ROI) was defined starting 10 slices from the end of the growth plate down 200 slices distal to the growth plate for bone volume/tissue volume

(BV/TV) analysis. BV/TV percentage values were recorded per mouse and averaged to give a mean BV/TV value per group (n=3/group).

Maxillae were scanned as described previously for linear bone loss measurements. DataViewer was used to orient maxillae with the CEJ of the first and second molars parallel in the sagittal and coronal planes. CTAn was used to define an ROI starting 10 slices apical from the CEJ and extending 50 slices apically. BV/TV percentages were recorded per mouse and averaged per group (n=3/group).

## **Histology**

Maxillae were decalcified in 15% ethylenediaminetetraacetic acid (EDTA) for four weeks with the solution changed 3x/week. CXCR3 KO and WT maxillae were embedded in paraffin and cut coronally in 5 $\mu$ m thick sections using a microtome (McBain Instruments, Chatsworth, CA, USA). Sections were stained with hematoxylin and eosin (H&E) to assess inflammatory infiltrate and Tartrate Resistant Acid Phosphatase (TRAP, Sigma Aldrich, MO, USA) to assess osteoclast counts. Cells that presented with  $\geq 2$  nuclei and were in contact with bone were considered to be osteoclasts. Osteoclasts were counted on six tissue sections per mouth and averaged to create a total osteoclast value per mouth (n=3 mice/group). Immunohistochemistry was performed using anti-Cox-2 (1:250, ab15191 Abcam, Cambridge, UK) to further assess general inflammation.

## **Statistics**



All statistical analyses were performed using Prism 5 GraphPad (CA, USA). For bone loss analysis, measurements were averaged per mouse and subsequently averaged per group to create a mean bone loss value per group (mean  $\pm$  standard error of the mean). For quantification of TRAP staining, osteoclasts were counted and averaged from  $n \geq 5$  slides per mouse. Mice were averaged to create a mean number of osteoclasts per group (mean  $\pm$  standard error of the mean). Significance levels were evaluated through a Student's *t* test. Significance levels were as follows:  $p \leq 0.05^*$ ,  $p \leq 0.01^{**}$ ,  $p \leq 0.001^{***}$ .

### **Study Approval**

This study (Animal Research Committee (ARC) protocol number 02-125) followed the guidelines according to the Chancellor's Animal Research Committee of the University of California, Los Angeles and the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) protocols for the submission of animal studies were followed (40).

## RESULTS:

CXCR3 KO mice showed statistically significant less bone loss compared to WT after 12 LPS injections. Radiographically, WT LPS treated mice showed severe loss of alveolar bone in between the first and second molars compared to CXCR3 KO LPS treated mice. Normalizing bone loss to WT, the LPS-injected KO showed ~50% reduction in bone loss compared to LPS-injected WT mice (Fig. 2A-C).

To confirm that the radiographic changes observed were due to LPS treatment and not due to inherent differences in bone quality between CXCR3 KO and WT mice, 3D volumetric analysis was performed. For both the maxillae and mesial trabecular bone distal from the growth plate in the femur, there was no statistically significant difference in BV/TV between CXCR3 KO and WT mice (Fig. 3A-D).

Histologically, H&E staining revealed an increase in cellular infiltrates in WT LPS treated mice compared to CXCR3 KO LPS treated mice. There was no difference in cellular infiltrates between the WT and CXCR3 KO control mice (Fig. 4A).

Assessment of protein expression of pro-inflammatory marker, COX-2, showed increased in staining in WT LPS treated groups compared to CXCR3 KO LPS treated mice. No overt differences in COX-2 expression were found between WT and CXCR3 KO control mice (Fig. 4B).

Quantification of osteoclasts through TRAP staining revealed statistically significantly more TRAP+ cells in WT LPS treated compared to CXCR3 KO LPS

treated mice. Additionally, WT control mice showed significantly more osteoclasts compared to CXCR3 KO control animals. When normalizing osteoclast numbers to alveolar bone length and surface area, WT LPS treated mice showed statistically significantly more osteoclasts per bone length and bone surface area compared to CXCR3 KO LPS treated mice (Fig. 5).

## DISCUSSION:

Periodontitis is a complex disease with many genetic and environmental influences. In order to investigate the genetic component while controlling confounding variables, a mouse model was used. In previous work, over 800 single nucleotide polymorphisms (SNPs) were identified as being associated to periodontitis using a GWAS approach. The Cxcl family, including genes Cxcl9 and Cxcl10, was selected for validation because in addition to the significant association in the GWAS, increased mRNA and protein expression were observed in the high bone loss strain compared to the low bone loss strain using microarray and immunohistochemistry. By deleting the CXCR3 receptor, we could evaluate how the absence of Cxcl9 and Cxcl10 affected LPS-induced periodontitis and thus begin to dissect the role of these chemokines in the immunoinflammatory response to bacteria.

Utilizing CXCR3 KO mice from a high bone loss phenotype strain, it was demonstrated approximately 50% less bone loss occurred following LPS injections compared to WT mice. This indicates that blocking the Cxcl9 and Cxcl10 pathway results in approximately 50% rescue of the periodontitis phenotype *in vivo*. Therefore, blocking CXCR3 may be a potential therapeutic target for periodontitis patients.

The CXCR3 receptor-ligand system is majorly involved in the chemotaxis of immune cells. CXCR3 is expressed on several immune cell types, including primarily Th1 cells, cytotoxic T-cells, and NK cells as well as dendritic cells and B cells (8,9,13).

Cxcl9 and Cxcl10, the CXCR3-binding chemokines, are induced in a wide variety of cells such as monocytes, endothelial cells, and fibroblasts (10), typically by pro-inflammatory cytokine IFN- $\gamma$ . Their production triggers the chemotaxis of immune cells bearing the CXCR3 receptor. While this mechanism is a part of normal host defense against infections, it may additionally contribute to an exacerbation of chronic inflammatory responses. This has been shown in many immunoinflammatory diseases including graft rejection, allergic reactions, liver disease, cardiovascular disease, and autoimmune diseases like type I diabetes mellitus, Graves' disease, rheumatoid arthritis, and systemic lupus erythematosus (10, 13, 21-27). We make the case that this mechanism additionally contributes to periodontal disease. Interestingly, periodontal disease is commonly linked to many of the previously listed conditions. It may be that there is a genetic overlap in susceptibility to these immunoinflammatory conditions.

Current clinical practice in the treatment of periodontal disease relies on the mechanical removal of oral biofilm (32). While specific pathogens are known to be more virulent than others, the complex structure of microbial biofilm, strongly adherent to the tooth surface, creates a protective matrix resistant to the actions of more targeted therapy like antibiotics (20). It has been shown the antibiotic concentration required to kill bacteria in biofilms compared to planktonic bacteria is 1000-15000x (20). While mechanical plaque removal is a generally effective treatment for periodontitis, the need for frequent recall intervals and our limited ability to detect active disease from previous destruction may result in the over or undertreatment of our patients. Additionally, while oral biofilm is necessary to

cause periodontitis, it is not sufficient as we see patients can present with the same microbial load but with varying disease severities (5). It is clear that the host immune response is a key factor in disease susceptibility. Therefore, modulation of the host response is an attractive therapeutic target for more individualized care. Several studies have investigated the adjunctive use of nonsteroidal anti-inflammatory drugs (NSAIDs) (33), COX-2 inhibitors (34), and bisphosphonates (35) in the treatment of periodontitis, showing mixed results with negative side effects.

Some studies have found an association of other cytokines and chemokines with periodontitis. It has been found that the expression of several CXCL family members and CXCL receptors were increased in periodontitis patients compared to healthy controls (28-31). However, little is known about the actual pathways and mechanisms of these signaling molecules. Accumulating evidence is showing the influence of dysregulated chemokine networks in chronic inflammatory diseases. CXCR3 and binding chemokines Cxcl9 and Cxcl10 are likely key players in the maintenance and amplification of inflammatory pathways. Therefore, CXCR3 may be an appropriate therapeutic target for periodontitis. Further research utilizing a CXCR3-antagonist is indicated to explore the therapeutic benefits with a more clinically applicable approach.

## CONCLUSION:

CXCR3 may be a possible target for modulating the host response in periodontitis susceptibility by dampening the inflammatory cascade following LPS-stimulation. Future work is needed to characterize the CXCR3 pathway and validate other candidate genes associated with LPS-induced bone loss. The ultimate goals are prevention by identifying patients at high risk for periodontal disease, as well as modulating disease progression with more targeted, individualized treatment.

FIGURES:

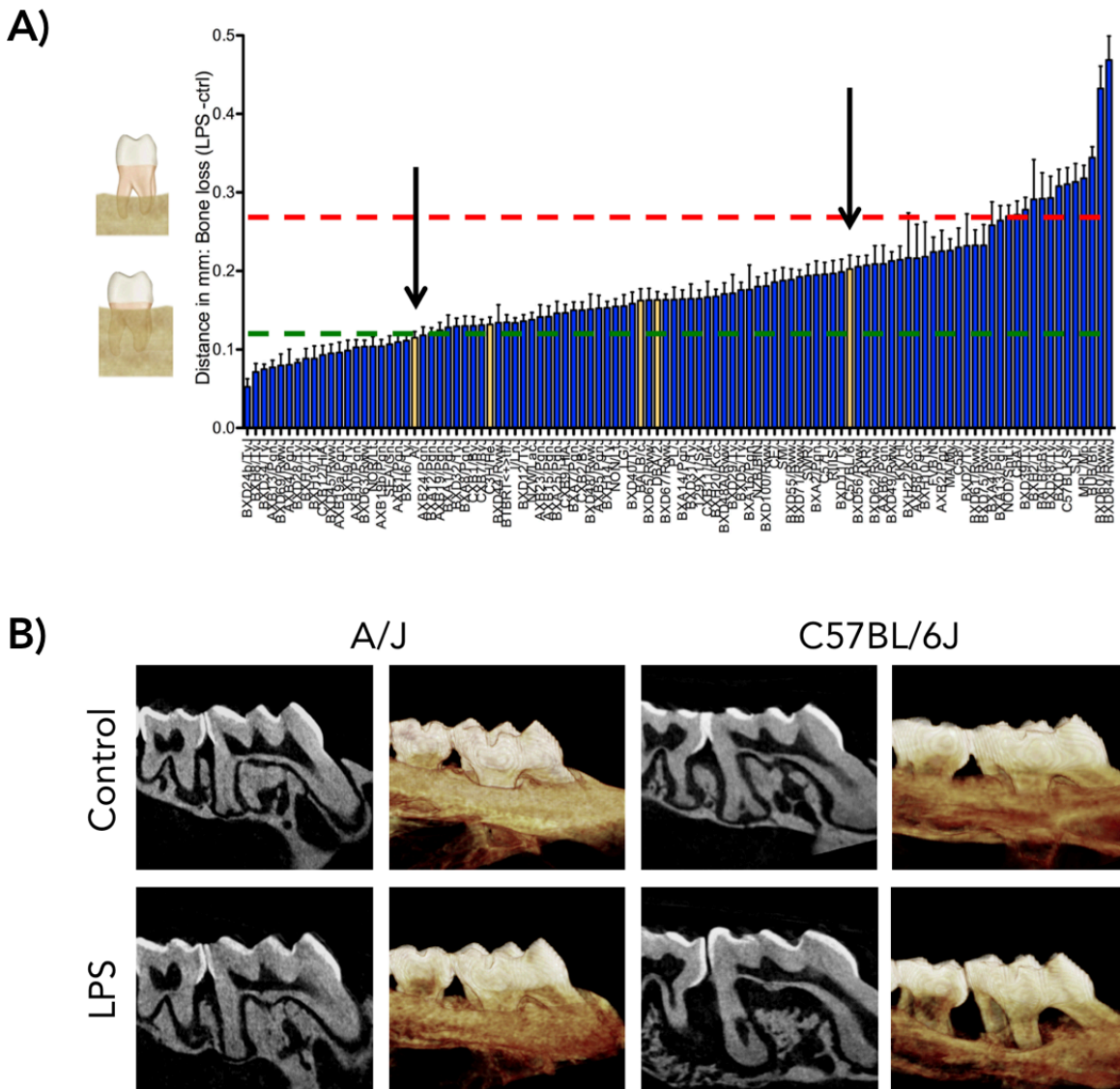


Figure 1: Hiyari et al. preliminary data. A) Graph representing bone loss in mm (LPS-ctrl) in 104 strains of the Hybrid Mouse Diversity Panel (HMDP)  $n \geq 6$  mice/strain. Data is represented in mean  $\pm$  standard error of the mean. The yellow bars represent the five parental strains of the HMDP with arrows highlighting resistant strain A/J (left) and susceptible strain C57BL/6J (right). The green bar indicates normal periodontal bone levels while the red bar indicates severe periodontal bone loss. B) Corrected sagittal and three-dimensional reformatted representative images of A/J and C57BL/6J LPS-injected mice.



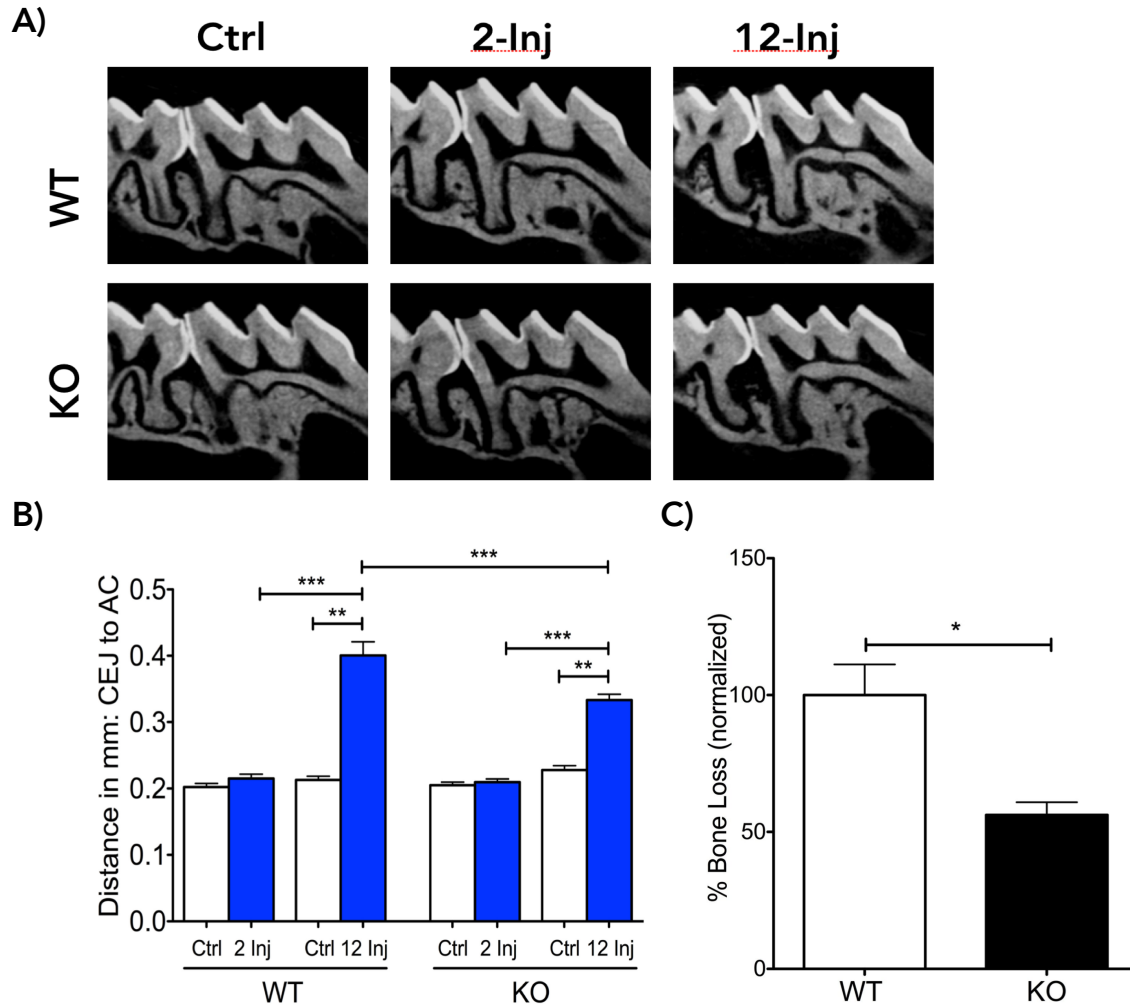


Figure 2: Deletion of CXCR3 *in vivo* causes a reduction in bone loss. A) Representative radiographic images of WT and Cxcr3-KO control and LPS treated mice. Note the increased bone loss in the WT LPS group compared to the KO LPS group after 12 injections. B) Graph representing the bone loss (LPS-ctrl) of WT and KO mice. C) Graph representing normalized bone loss in LPS treated WT and KO mice. For both graphs (B and C), significance was compared using a Student's *t* test.  $n=3$  mice/group with 4 measurements per mouse,  $p \leq 0.05^*$ ,  $p \leq 0.01^{**}$ ,  $p \leq 0.001^{***}$ . Data represented as mean  $\pm$  standard error of the mean.

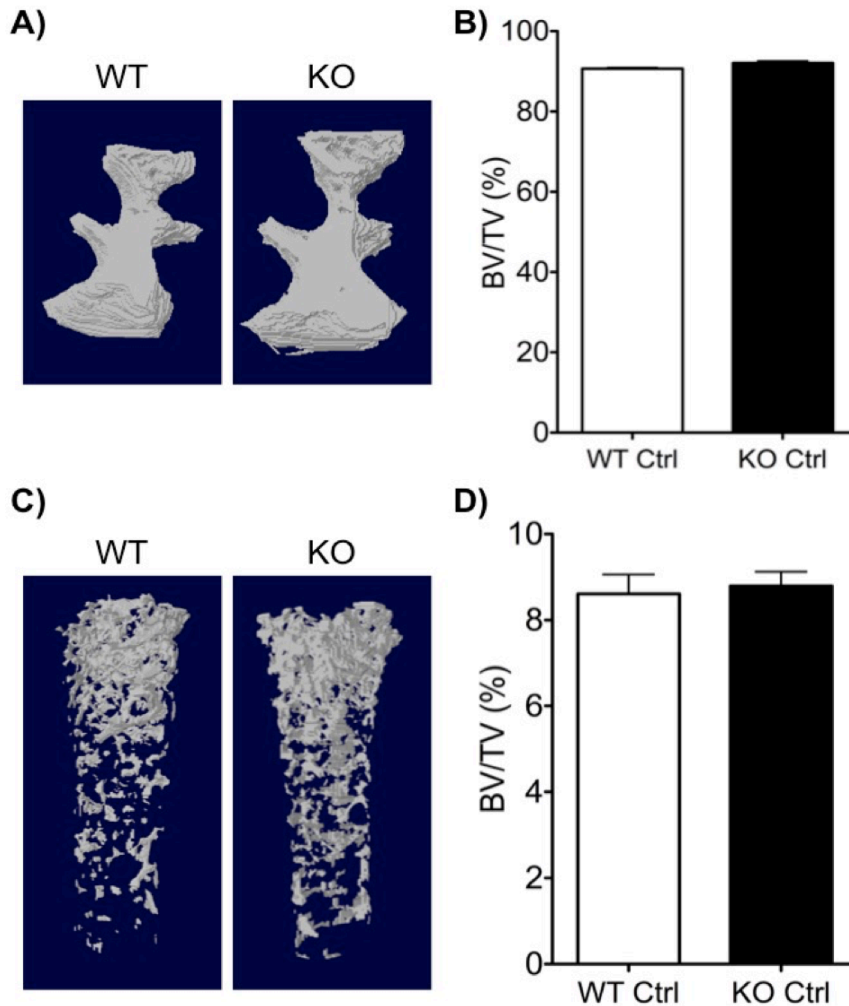


Figure 3: Radiographic assessment of bone volume/tissue volume (BV/TV) in WT and CXCR3 KO mice. A) Representative volumetric 3D reconstruction of maxilla in WT and CXCR3 KO mice. The area represented is between the first and second molars. B) Graph representing % BV/TV in WT control and CXCR3 KO mice. C) Representative volumetric 3D reconstruction of the mesial femur distal from the growth plate of WT control and CXCR3 KO mice. D) Graph representing % BV/TV in WT control and CXCR3 KO mice. For both graphs (B and D), significance was compared using a Student's *t* test.  $n=3$  mice/group,  $p \leq 0.05^*$ ,  $p \leq 0.01^{**}$ ,  $p \leq 0.001^{***}$ . Data represented as mean  $\pm$  standard error of the mean.

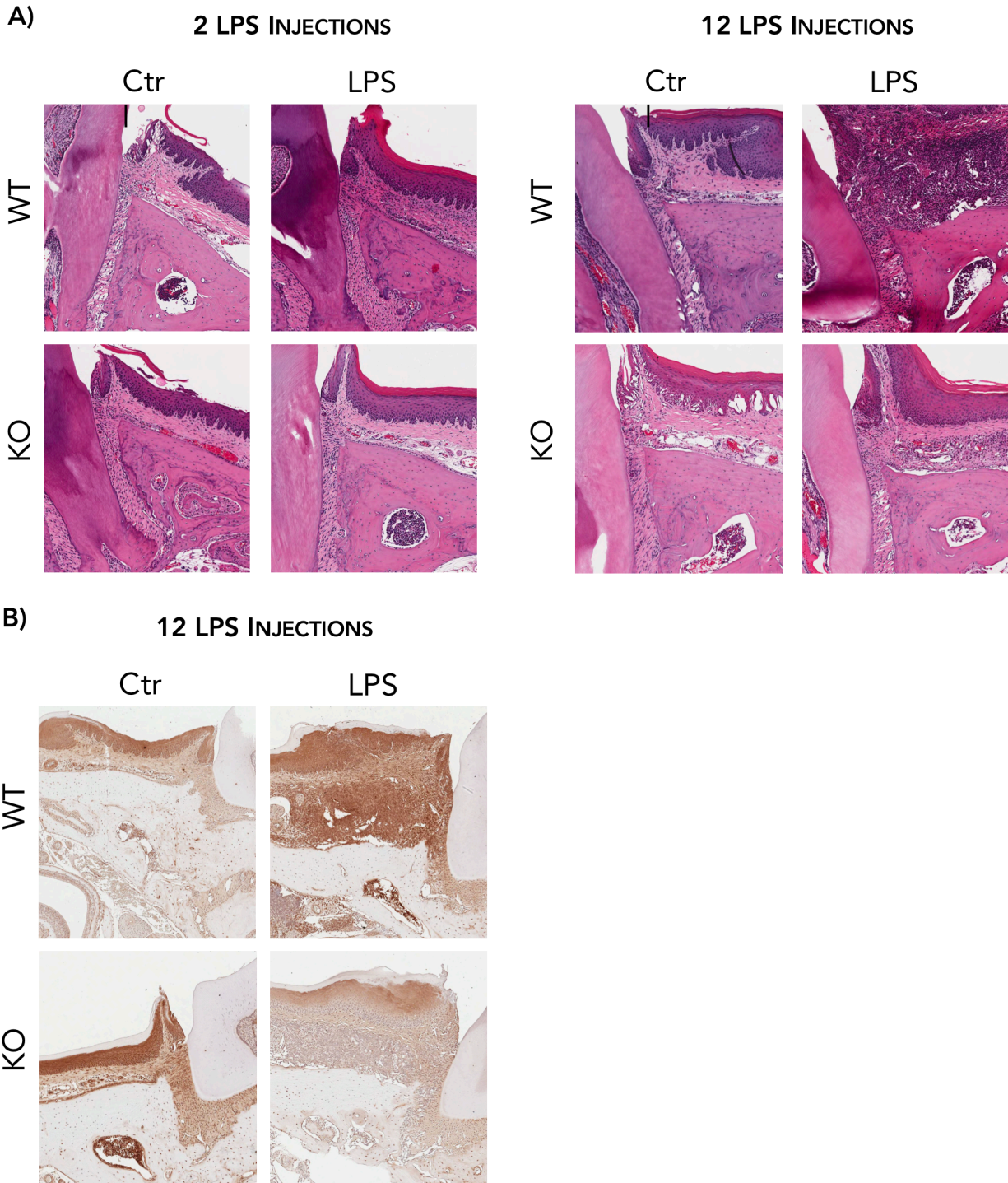


Figure 4: Histological assessment of inflammation in WT and CXCR3 KO mice. A) Hematoxylin and eosin (H&E) stained tissue sections of WT and KO control and LPS treated groups. Increased inflammatory infiltrates visualized in the WT LPS group, starting at the 2-injection time point and increasing dramatically at the 12-injection time point. B) COX-2 immunostaining in WT and KO control and LPS treated

groups. Increased COX-2 expression (brown stain) is visualized in the WT LPS group, indicated increased presence of inflammation.

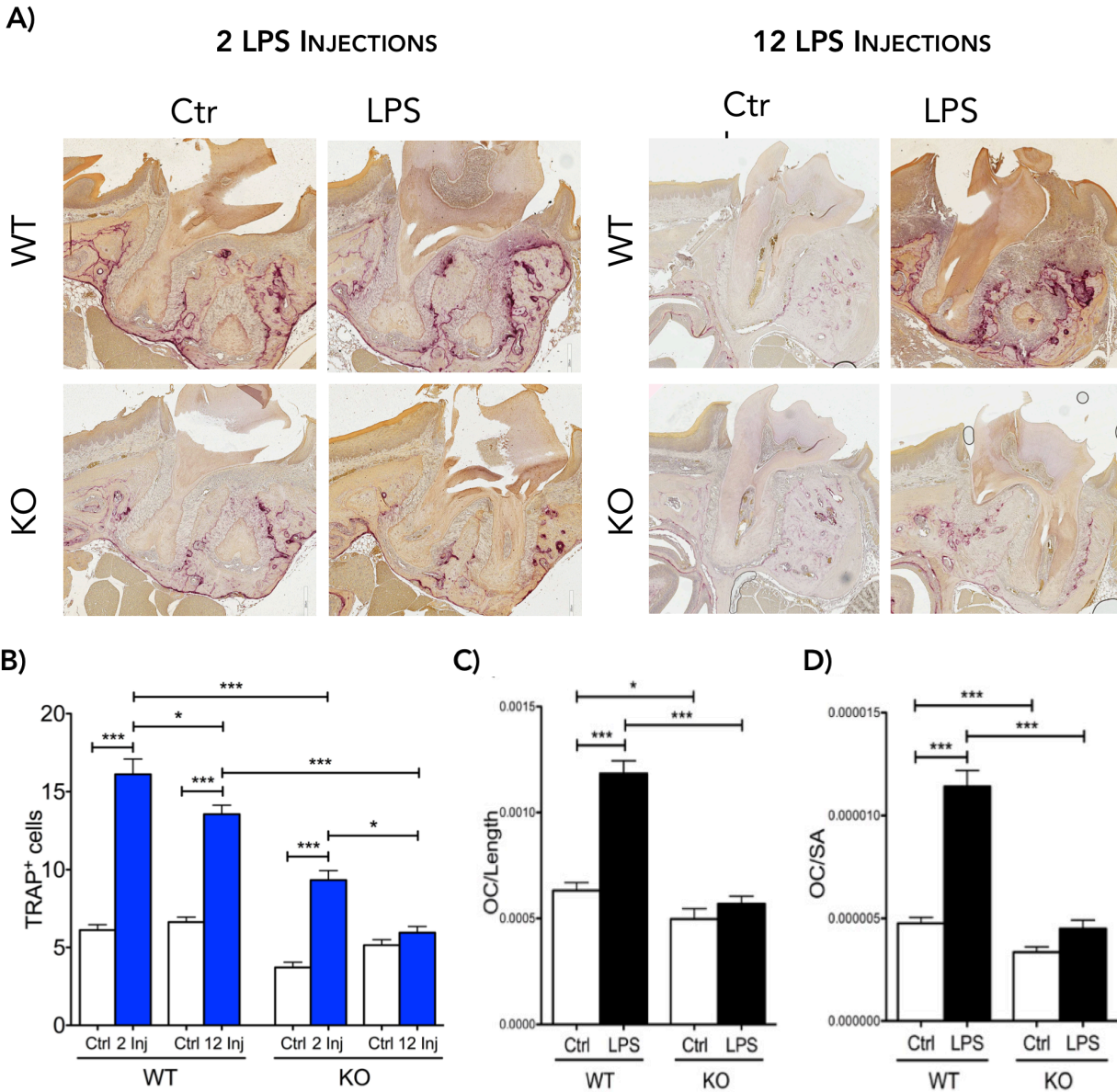


Figure 5: Histological assessment of osteoclast numbers in WT and CXCR3 KO mice. A) Tartrate Resistant Acid Phosphatase (TRAP) staining for osteoclasts. Note the increase in TRAP+ cells in WT LPS treated mice compared to KO LPS treated mice at both the 2-injection and 12-injection time points. 20X magnification. B) Graph representing total number of averaged osteoclasts in WT and CXCR3 KO control and LPS groups. C) Graph representing osteoclast numbers divided by the length of alveolar bone measured. D) Graph representing osteoclast numbers divided by the surface area (SA) of the alveolar bone considered in analysis. For all graphs (B, C, D), significance was compared using a Student's *t* test. *n*=3 mice/group with 6

slides analyzed per mouse,  $p \leq 0.05^*$ ,  $p \leq 0.01^{**}$ ,  $p \leq 0.001^{***}$ . Data represented as mean  $\pm$  standard error of the mean.

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