The Contribution of Macrophages in Old Mice to Periodontal Disease

D. Clark[1], B. Halpern[1], T. Miclau[2], M. Nakamura[3,4], Y. Kapila[1], R. Marcucio[2].

1.
University of California San Francisco
Orofacial Sciences
513 Parnassus Ave
San Francisco, CA, USA 94143

2.
University of California San Francisco
Orthopaedic Trauma Institute
2550 23rd Street
San Francisco, CA, USA 94110
415-206-5366

3.
University of California San Francisco
School of Medicine
533 Parnassus Ave.
San Francisco, CA, USA 94117

4.
San Francisco Veterans Affairs Health Care System
Arthritis/Immunology Section
4150 Clement St. 111R
San Francisco, CA, USA 94121

Expanded Methods

Periodontal disease model

All animal procedures were approved by the UCSF Institutional Animal Care and Use Committee and were carried out in strict accordance with the guidelines of the US National Institutes of Health for the care and use of Laboratory animals. Old (24 month) and young (3 month) male C57BL/6 mice were obtained from the Aging Rodent Colony of the National Institute on Aging. 34 mice were used in total. Periodontal disease induction was accomplished by first anesthetizing the mice with a 1:1 solution of dexmedetomidine and ketamine delivered IP. A 6.0 silk suture inoculated with *Porphyromonas gingivalis* (ATCC 33277) was tied in a subgingival position around the second maxillary molars bilaterally, as previously described (Abe and Hajishengallis 2013). Ligature models of periodontal disease induce a robust osteolytic inflammatory response that is more acute in nature compared to the natural chronic inflammatory processes in human periodontal disease. The sutures remained in place for 7 days to adequately induce periodontal disease. Control mice in both age groups received a sham surgery where mice were anesthetized, a sterile suture was placed subgingivally and then immediately removed. At the end of the 7-day induction period, mice were euthanized, or in a second arm of the study, the sutures were removed under anesthesia and the animal was allowed to recover for an additional 7 days. At the end of disease induction or disease recovery period, animals were euthanized, and maxillae were collected for analysis. One half of the maxilla was isolated and prepared for histological analysis. In the other half of the maxilla, the gingiva was removed and prepared for qRT-PCR analysis while the remaining alveolar bone and teeth were prepared for micro-CT analysis. All mice met the intended experimental end point and none were excluded from analysis.

Macrophage depletion

During disease induction or disease recovery, PLX3397 (Plexxikon, Berkeley, CA) was administered to half the mice in each age group to deplete macrophages. PLX3397 is a small

molecule inhibitor of the macrophage colony stimulating factor 1 receptor (CSF1R) (Butowski et al. 2016). Inhibition of CSF1R prevents monocyte differentiation into mature macrophages. Inhibition of FMS-like tyrosine kinase 3 (FLT3) and proto-oncogene receptor c-KIT by PLX3397 has also been reported (Butowski et al. 2016). PLX3397 was prepared in the mouse chow and was delivered *ad libitum* throughout the 7-day induction or recovery period at an average dose of 40mg/kg/day. Non-treated groups received the control chow provided by the drug manufacturer.

Micro-CT analysis

Bone volume/total volume (BV/TV) and bone mineral density (BMD) of the alveolar bone was quantified as a measure of disease severity. One half of the maxilla containing the 3 molar teeth was isolated, defleshed, and soaked in 30% hydrogen peroxide overnight, and fixed in 10% buffered formalin. Samples were scanned using Scano Medical μCT (SCANCO Medical AG, Brüttisellen, Switzerland) set at 55kVp and 109μA with a slice thickness of 5μm. Analysis of the scanned images was performed using Scanco Medical μCT Evaluation Program v6.5. The region of interest was delineated along transverse slices isolating the alveolar bone supporting the teeth and excluding tooth structure. Mesial and distal boundaries were made at the buccal furcation of the first molar and the buccal furcation of the third molar.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Inflammatory cytokine expression within the gingiva was quantified and compared across age and treatment groups. Gingiva was isolated from one half of the isolated maxilla via blunt dissection off the bone with care to remove all interdental soft tissue. The isolated gingiva was prepared for qRT-PCR analysis. Briefly, gingiva was homogenized in Trizol and mRNA was isolated. cDNA was reverse transcribed using Superscript III (Invitrogen). qRT-PCR was performed with SYBR Green and the following primers; GAPDH (F: 5'-AGCCTCGTCCCGTAGACAAAAT-3'; R: 5'-CCGTGAGTGGAGTCATACTGGA-3'), IL-1β (F: 5'-TGTAATGAAAGACGGCACACC-3'; R: 5'-TCTTCTTTGGGTATTGCTTGG-3'), TNFα (F: 5'-

TGCCTATGTCTCAGCCTCTTC-3'; R: 5'-GAGGCCATTTGGGAACTTCT-3'), IL-6 (F: 5'-TCCAATGCTCTCCTAACAGATAAG-3'; R: 5'-CAAGATGAATTGGATGGTCTTG-3'), IL-17 (F: 5'-GCTCCAGAAGGCCCTCAGA-3'; R: 5'-CTTTCCCTCCGCATTGACA-3'). Gene expression was normalized to the house keeping gene GAPDH and presented as relative gene expression ($\Delta\Delta C_T$) or fold change ($2^{-\Delta\Delta CT}$). GAPDH demonstrated stable expression across age groups (Supplemental Table 1).

Tissue embedding

One half of the maxilla was isolated and fixed in 4% paraformaldehyde for 24 hours. The tissue was decalcified with 19% ethylenediaminetetraacetic acid (pH 8) for 28 days with the solution changed every other day. Samples were embedded in optimal cutting temperature compound (Tissue-Tek, Sakura Finetek, CA, USA). Frozen serial sections were cut at a thickness of 8µm on a cryotstat.

Immunohistochemistry

Quantification of macrophages within the periodontium was performed via immunohistochemical staining for F4/80+ macrophages. Frozen sections were first blocked with 3% H_2O_2 for 45 minutes followed by antigen retrieval with Trypsin 0.1% in dH_2O for 30 minutes. Sections were then blocked with 5% goat serum albumin for 1 hour. The primary antibody, rat anti-mouse F4/80 (BD Biosciences), was incubated with the section at a 1:200 dilution in 5% goat serum overnight at 4°C. The secondary anti-body, HRP conjugated goat anti-mouse IgG, was applied at 1:500 dilutions and incubated for 1 hour. Secondary antibody signal was amplified using the VectaStain ABC Kit (Vector) and finally stained with 3,3'-diaminobenzidine (DAB) substrate. One representative section was selected per animal, and 3 regions of interest (ROI) per section were imaged with a light microscope at 400x for analysis. The ROIs were adjacent to the tooth and included the gingival epithelium and connective tissue, periodontal ligament space, and alveolar

bone. F480+ macrophages were quantified within each ROI using ImageJ (National Institutes of Health) imaging software by a blinded examiner. The 3 ROIs were averaged per mouse.

TRAP staining

Quantification of osteoclasts within the periodontium was performed via tartrate-resistant acid phosphatase (TRAP) staining using the acid phosphatase leukocyte kit (Sigma-Aldrich). One representative TRAP-stained section was selected per animal, and 3 ROIs per section were imaged at 400x for analysis. Analysis of the number of TRAP+ multinucleated osteoclasts per millimeter of bone surface (N.Oc/BS) and the percentage of TRAP+ bone surface over total bone surface (Oc.S/BS) was completed within each ROI using ImageJ imaging software by a blinded examiner according to the histomorphometric analysis standards of the American Society for Bone and Mineral Research (Dempster et al. 2013) . The 3 ROIs were averaged per mouse.

Linear bone loss measurements

In healthy control young and old mice, one half of the maxilla was isolated. The gingiva was removed and analyzed via RT-qPCR. The remaining alveolar bone and teeth were soaked in 30% hydrogen peroxide overnight and defleshed completely. The hemimaxillae were stained with 1% methylene blue for 5 minutes prior to obtaining measurements. Magnified images of the maxilla were captured using a dissecting microscope. One image of the buccal aspect and one image of the lingual aspect of the maxilla were captured for each sample. Bone loss measurements were made using ImageJ. Each image also captured a ruler laid adjacent to the sample to provide a consistent marking to calibrate the measurement tool within ImageJ. Bone loss was measured from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) at 6 sites per tooth (3 sites on the buccal and 3 sites on the lingual surface of each tooth). All measurement sites were averaged per animal.

Statistical Analysis

The BV/TV, BMD, linear bone loss, and cell quantification were calculated per sample and presented as mean ± standard deviation (SD). Groups were first analyzed via ANOVA for significant differences, and between group differences were analyzed using a two-tailed t test. qRT-PCR utilized technical triplicates and the mean QT value was calculated. Relative gene expression ($\Delta\Delta C_T$) was calculated and presented as mean ± standard error of the mean (SEM) and analyzed using an ANOVA followed by between group comparisons using a two-tailed t test. Significance for all analysis was determined at $p < 0.05$. Sample size was determined using a power analysis to obtain a power of 80% to detect a 5% change in BV/TV using a type 1 (alpha) error rate of 0.05. All statistical analysis was performed using GraphPad Prism v.7 software.

Supplemental Table 1: GAPDH validation across age groups and conditions.

Gene	Condition	Ct value-mean	CT value-SD	student t-test
GAPDH	Control gingiva_Old	23.8	1.3	p=.07
	Control gingiva_Young	25.56	1.4	
	Periodontal disease induction_Old	24.34	1.62	p=.99
	Periodontal disease induction_Young	24.35	1.14	
	Periodontal disease induction_Old	24.34	1.62	p=0.46
	Periodontal disease induction+PLX3397_Old	23.65	2.04	
	Periodontal disease induction_Young	24.35	1.14	p=.62
	Periodontal disease induction+PLX3397_Young	24.91	1.34	