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Biological

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NOD2 Contributes to *Porphyromonas gingivalis*–induced Bone Resorption

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APPENDIX

Infection with *Porphyromonas gingivalis*

The *Porphyromonas gingivalis* W83 was grown in prereduced brain heart infusion (Difco, Sparks, MD, USA), supplemented with 5% sheep blood (Cecon), 1 $mg{\cdot}mL^{-1}$ hemin, and 0.5 $mg{\cdot}mL^{-1}$ menadione (Sigma-Aldrich, St Louis, MO, USA), and incubated in an anaerobic glove box (85% N_2, 5% CO_2, 10% H_2) at 37°C for 2 d. Before oral infection, all animals received antibiotic treatment—Bactrim suspension (concentration of sulfamethoxazole/trimethoprim oral suspension is 400/80 mg/5 mL), 15 mL / 300 mL of drinking water—for 4 d to reduce the indigenous oral flora, followed by 3 d of an antibiotic-free period.

In the 4th week after the last inoculation, sterile swab was placed in the oral cavity of mice and swirled for 15 sec before being placed in 1 mL of phosphate buffered saline. Bacterial infection was confirmed by culture of the sample on blood agar plates under anaerobic conditions, and CFU/mL was counted.

Immunofluorescence

Osteoclast precursors cells were plated in glass slides inside 24-well plate (2×10^5 cells/well) and cultured in presence of M-CSF and RANKL for 24 hr. After this time, preosteoclasts were incubated with *P. gingivalis* (multiplicity of infection at 1) stained with CFSE (5 μM) for 30 min. Preosteoclasts were incubated with *P. gingivalis* for 1 hr. Noninternalized bacteria was removed, and cells were fixed with paraformaldehyde (1%) and subsequently stained with phalloidin-rhodamine (Invitrogen). Slides were analyzed by confocal microscopy.

Flow Cytometry Analysis

P. gingivalis were stained with Fluorescein Isothiocyanate isomer 1 (Sigma F 7250; 0.1 mg/mL in 0.1M sodium bicarbonate buffer) for 1 hr. Preosteoclasts were incubated with *P. gingivalis* (multiplicity of infection at 3) for 30 min. Noninternalized bacteria was removed, and cells were analyzed by FACSCalibur using CellQuest software (BD Biosciences).

Primers

Primers and probes (Applied Biosystems) included RANKL (Mm00441906), OPG (Mm01205928), cathepsin K (CTSK) (Mm00484039), and MMP-9 (Mm00442991). The gene beta-actin (Mn00607939) or GAPDH (Mm99999915) was used as the endogenous gene. The relative levels of gene expression were determined by the $2^{-\Delta\Delta \text{ Cycle Threshold}}$ ($2^{-\Delta\Delta CT}$) method, in which data are shown as fold increase over the control group (noninfected).