SUPPLEMENTAL MATERIAL

Supplemental Methods

Bacterial strain and growth conditions. *T. denticola* (10^9 cells per mL: grown in GM-1 broth for 48–72 h as a log phase culture) was mixed with equal volumes of sterile 4% (w/v) low-viscosity carboxymethylcellulose (CMC: Sigma, St. Louis, MO, USA) with PBS for oral infection inocula.

Mouse infection. Eight-week-old male $ApoE^{null}$ mice (The Jackson Laboratories, Bar Harbor, ME) were kept in groups and housed in microisolator cages. Mice were fed standard chow and water *ad libitum* and were randomly distributed into two groups before infection (Group1, *T. denticola* infection, n=24 mice. Group 2, sham-infected control, n=24 mice). Mice were administered kanamycin (500 μg/ml) for 3 days in the drinking water and the oral cavity was swabbed with 0.12% chlorhexidine gluconate (Peridex: 3M ESPE Dental Products, St. Paul, MN) mouth rinse to suppress the normal oral microbiota. *T. denticola* 10^9 cells were administered orally to mice for 4 days per week on every third week for eight infections. Oral plaque/microbial samples were taken from isoflurane anesthetized mice post-infection in 100μl TE (Tris-EDTA) buffer.

Serum antibody analysis. To provide additional confirmation of oral polymicrobial infection and to document an immunological response to *T. denticola* infection, we evaluated levels of the spirochete specific IgG and IgM against formalin-killed whole cells for *T. denticola* in sera from both *T. denticola*-infected and sham-infected mice. Briefly, diluted infected mouse serum (1:100) was incubated in wells of *T. denticola*-coated microtiter polystyrene plates (Costar, Corning, NY, USA) for 3 h at room temperature. After washing, alkaline phosphatase-conjugated goat anti-mouse IgG (1:5000) and IgM (1:5000) (Bethyl Laboratories, TX, USA) were added and

incubated for an additional 2 h at room temperature on a rotator. The substrate (p-nitrophenylphosphate; Sigma, 1mg/ml) was added to the washed plates, and the reaction was terminated by using 3M NaOH. The optical density (OD) was measured at OD_{405} using a Bio-Rad Microplate Reader.

Morphometric Analysis of Horizontal Alveolar Bone Resorption. The 12- and 24-week microbial infected and control jaws (n=9 mice) were immersed in 3% (vol/vol) hydrogen peroxide over night and stained with 0.1% (wt/vol) methylene blue to delineate the cemento-enamel junction (CEJ) and alveolar bone crest (ABC) using a Zeiss SteREO Discovery.V8 microscope and Zeiss AxioVision software version 4.8.2. Alveolar horizontal area bone resorption was determined by comparing the area between the CEJ and ABC in infected to sham-infected mice. Intrabony defect was determined by the presence or absence on the palatal and buccal sides of all three molars, and the percent was determined by dividing the number of intrabony defects by the total number of sites. Morphometric measurements represent the average alveolar bone resorption determined by three independent viewers blinded to the mice groups.

Histomorphometric Analysis of Aortic Atherosclerotic Plaque. Hearts and aortas harvested at euthanasia of mice were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5 um sections for Haematoxylin and Eosin (H&E) staining. The aorta at the level of the aortic valve, where atherosclerotic plaque predominates in $ApoE^{null}$ mice, and in the ascending aorta was examined for plaque by cutting six up to eleven 5µm sections at 50-100 micron length intervals along the aorta from the ascending aorta to the level of the aortic valve, allowing confirmation of presence or absence of atherosclerotic plaque (12-22 sections per aorta per mouse). The two sections with largest plaque areas for each mouse aorta were used for all

subsequent analyses ensuring that the largest plaques detected were used for all subsequent analysis. The aortic arch and descending thoracic and abdominal aorta were also sectioned but had minimal if any visible plaque. Plaque area and also intimal and medial thickness were measured, and/or calculated, by two blinded viewer's using an Olympus DP71 microscope and ImagePro MC 6.0 software, with calibration to microscopic objective. Plaque area and calculated intimal/medial thickness ratios provide differing measures of atherosclerotic plaque size, intimal/medial thickness ratios providing a correction for potential significant variations in aortic size and diameter at differing aortic anatomic levels.

Fluorescence *in situ* hybridization (FISH). The protocol was modified from Fuchs 2007 (1) and Mantz 1999 (2). Tissue sections on slides were deparaffinized in decreasing concentrations of xylene and ethanol. Samples were blocked with Denhardt's reagent (Fisher) and covered with hybridization solution (900mM NaCl, 20mM Tris-HCl pH 7.5, 0.01% SDS, 20% formamide) containing 5μg/ml probe, and incubated for 3 hours at 50^{o}C. The probe was rinsed off and incubated in wash buffer (20mM Tris-HCl, pH7.5, 5mM EDTA, 0.01% SDS, 0.225M NaCl), at 48^{o}C for 25 min. Blocking buffer, hybridization buffer, and wash buffer all contained protectRNA (Sigma-Aldrich) to protect bacterial RNA from degradation. Tissues were counter-stained with DRAQ5 and mounted in mowiol. Slides were dried overnight before being viewed with a fluorescence microscope. Images were taken using a spinning disk confocal system connected to a Leica DMIRB microscope with a 63× oil immersion objective equipped with a Photometrics cascade-cooled EMCCD camera under the control of the open-source software package μManager (http://www.micro-manager.org/). Confocal images were processed using ImageJ software (NCBI).

Determination of serum amyloid A (SAA) in ApoE^{null} mice. The SAA ELISA kit contained pre-coated plates containing bound anti-SAA antibody that was used both for calibrations and samples. All procedures were performed at room temperature. Diluted (1:100) serum samples were added to pre-coated plate and incubated for 1 h. After the wells were washed with a washing solution from the kit, Enzyme-Antibody Conjugate was added and plate was incubated for 30 min. After another wash TMB substrate solution from the kit was added and incubated for 10 min. The reaction was stopped using the stop solution contained in the kit. Absorbance was determined at OD_{450} using a Bio-Rad Microplate Reader. A second order polynomial calibration curve was made using the standards contained in the kit and the data were analyzed for statistical significance by Mann-Whitney t-test.

Determination of serum lipoproteinin ApoE^{null} mice. Sera were obtained from the blood collected from the cardiac puncture of each mouse at the time of euthanasia and used to determine serum lipoproteins (chylomicrons, VLDL, LDL, HDL), serum cholesterol, and triglyceride profiles by high-pressure liquid chromatography which is based on the gel permeation principle using the method of Liposearch at skylight biotech, Japan.

Mouse atherosclerosis RT^2 Profiler PCR array. Atherosclerosis-related gene profiles were evaluated using RT^2 Profiler Mouse Atherosclerosis PCR Array. Briefly, tissues were mechanically disrupted using homogenized using a mechanical tissueRuptor (QIAGEN, Valencia, CA) and RNA was extracted using an RNeasy kit (QIAGEN, Valencia, CA), followed by reverse transcription with the RT^2 First Strand Kit (QIAGEN, Valencia, CA). RNA samples were prepared for array with the SYBR Green Master mix (QIAGEN, Valencia, CA). QPCR Cycling was performed according to the manufacturer's protocol, and data were analyzed using the PCR Array Data Analysis V4 excel worksheet.

Evaluation of inflammatory cytokine array in mouse serum. Inflammatory cytokines in mouse serum were evaluated using a Ray Biotech Mouse Inflammatory Cytokine glass chip Array. Fluorescent signals were detected using a GenePix 4400 scanner.The RayBio Analysis tool excel sheet was used for analysis. Fold difference in expression was plotted vs. median fluorescence intensity (MFI) value of sham-infected mice for each cytokine and important clusters of cytokines up- or down-regulated were determined based on the position in the graph and interactions with other affected genes.

Statistical Analysis. Graph Pad Prism software v.5 was used to perform statistical analyses of bacterial-specific serum antibody levels, serum lipid profiles, SAA, NO, and alveolar bone resorption by a two-tailed Student's t test. The alveolar bone resorption and antibody analysis data are presented as means ± standard deviations. Aortic plaque histology measurements were analyzed by analysis of variance (ANOVA) with the Statview program and *post hoc* Fisher's PLSD analysis, with bar graphs represented as mean ±standard error. In all analyses, *p* values less than 0.05 were considered statistically significant.

Supplemental References

1. **Fuchs BM, Pernthaler J, Amann R**. Single Cell Identification by Fluorescence *In Situ* Hybridization. 2007. Methods for General and Molecular Microbiology (3rd Edition). 886–896.
2. **Manz W.** 1999. *In situ* Analysis of Microbial Biofilms by rRNA-Targeted Oligonucleotide Probing. Methods in Enzymology. **310**: 79–91.

Table 1. Distribution of oral microbial samples positive for *T. denticola* by PCR

Bacterial infection	Positive oral microbial sample for bacterial genomic DNA

Sampling *	1	2	3	4	5	6	7	8
T. denticola infected†	4	12	0	0	11	0	11	11
Sham-infected‡	0	0	0	0	0	0	0	0

* Number of mice sampled from 1-4th sampling was 24, while number of mice sampled from 5-8th sampling was 12. †Mice were infected with *T. denticola* for 8 alternate weeks. Samples were analyzed using *T. denticola* species-specific primers with positive and negative controls. ‡Oral microbial samples were collected from sham-infected control mice periodically and examined for *T. denticola* using bacteria-specific primers and all mice were negative.