Supplemental data

***Porphyromonas gingivalis* Accelerates Inflammatory Atherosclerosis in the Innominate Artery of ApoE Deficient Mice**

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Methods

Bacterial Challenge and Immunization

Mice in group ii (n=6) and group iv (n=6) were immunized subcutaneously two times a week for 3 weeks with 100μl (~1.0 x 10^9 CFU) of heat killed *P. gingivalis* strain 381 in PBS. Mice in group i (n=6) and iii (n=6) were injected subcutaneously with 100μl of PBS and served as non-immunization groups as previously described. Prior to oral challenge with *P. gingivalis* mice received 4% sulfamethoxazole in their drinking water for 2 wk to clear their normal oral micro flora. Mice in group iii and iv were oral challenged with *P. gingivalis* strain 381 (~1.0 x 10^9 CFU / mouse) with 2% carboxymethyl cellulose and mice in group i and ii were oral challenged with PBS with 2% carboxymethyl cellulose five times a week for three weeks as previously described. Mice were imaged at 11, 17, and 25 wk after infection. At 34 wk of age (25 wk after infection), mice were sacrificed after imaging, the innominate arteries and spleens collected, and cross-sections obtained from the innominate artery. Spleen size was determined according to the ratio of spleen weight divided by body weight.

Other MRA parameters

slab thickness = 1.5 cm, flip angle = 45°, repetition time (TR) = 20 ms, echo time (TE) = 2.2 ms, field of view (FOV)= 1.5 x 1.5x 1.5 cm, matrix = 128 x 128 x 128, in-plane, number of average (NEX) = 4.

Other MRI parameters

slice thickness = 0.5 mm, TR = 200 ms, TE = 4.7 ms, FOV = 2.5 x 2.5 cm, matrix = 256 x 256, in-plane resolution = 0.098 x 0.098 mm , NEX = 16.

Polarized light microscopy to study the melting behavior of lipids in atherosclerotic plaques

The slides were heated and cooled from 20 to 60 °C with a rate of 1-2 °C / minute. Digital photographs of the tissue were taken at different temperatures. The onset of melting was defined when the intensity of the birefringence started to decrease and the completion when the birefringence completely disappeared, indicating a liquid-crystal to an isotropic liquid-transition. Any crystalline material that remained birefringent at 60 °C and had a needle or plate-like appearance was identified as cholesterol monohydrate crystals. Subsequently, the sections were cooled to 20 °C and the temperatures at which the birefringent patterns began to reappear indicated an isotropic to liquid-crystalline (cholesteric or smectic) transition.

Plasma Analysis for Systemic Markers

The plasma was separated by centrifugation and stored at -80 °C until analysis. Samples were run in duplicate. Acquisition conditions were set with a minimum of 100 beads per analyte. Raw data (mean fluorescence intensity) from all the bead combinations were analyzed using MasterPlex® QT software (Hitachi Software Engineering America, Ltd.) A four-parameter curve fit was applied to each standard

curve in order to obtain sample concentration values. The minimum detectable concentrations for each cytokine were as follows: <1 pg / ml (IFN-γ), 5 pg / ml (TNF-α), <10 pg/ml (IL-1α, IL-12 p40/p70), 10 pg/ml (IL-1β, IL-6, GM-CSF). Values for all plasma samples were included in the analysis. Data was analyzed for statistical significance between groups by Mann Whitney U test. To validate the multiplex results, plasma samples were analyzed by ELISA (in duplicate) for IL-6 and TNF-α. The levels of IL-6 in plasma collected at sacrifice were similar between the two methods. ELISAs were also performed to monitor the levels of IL-6 and TNF-α at various stages of the experiment, including prior to immunization, post immunization, and after the last oral challenge.

Legends to Supplemental Figures

Supplemental Figure 1. ApoE$^{-/-}$ mice were immunized prior to *P. gingivalis* challenge as described in the Materials and Methods. MRI images were taken at 11, 17, and 25 weeks after the start of bacterial challenge.

Supplemental Figure 2. iNOS and Arg-I expression were detected in the innominate artery.

Representative images of plaque sections. The iNOS expression (**A**-**I**) and Arg-I expression (**J**-**R**) were detected immunohistochemically in the innominate artery. (**A, F, J, and O**) group i: uninfected / non-immunized, (**B, G, K, and P**) group ii: uninfected / immunized, (**C, H, L, and Q**) group iii: *P. gingivalis* infected / non-immunized, (**D, I, M, and R**) group iv: *P. gingivalis* infected / immunized ApoE$^{-/-}$ mouse, and (**E and N**) Isotype control. **F, G, H, I, O, P, Q, and R** are the magnified images in the boxes in **A, B, C, D, J, K, L, and M,** respectively. Bar represents 200 μm (**A**-**E and J**-**N**) and 50 μm (**F-I and O-R**). Arrows point the positive staining.

Supplemental Figure 3. F4/80, iNOS, Arg-I and T cell accumulation in the spleen following *P. gingivalis* infection. F4/80 (**A-E**), iNOS (**F-J**), Arg-I (**K-O**), and CD3 (**P-T**) expression in the spleens of representative mice were determined. (**A, F, K, and P**) group i: uninfected / non-immunized, (**B, G, L, and Q**) group ii: uninfected / immunized, (**C, H, M, and R**) group iii: *P. gingivalis* infected / non-immunized, and (**D, I, N, and S**) group iv: *P. gingivalis* infected / immunized ApoE$^{-/-}$ mouse, (**E, J, O, and T**) isotype control. Bar represents 50 μm. **(U)** F4/80^{+} cell count in the spleen. In non-immunized ApoE$^{-/-}$ mice *P. gingivalis* infection increased the number of F4/80^{+} cells as compared to uninfected ApoE$^{-/-}$ mice. In *P. gingivalis* infected ApoE$^{-/-}$ mice, the number of F4/80^{+} cells was significantly decreased in immunized ApoE$^{-/-}$ mice as compared to non-immunized ApoE$^{-/-}$ mice. **(V)** CD3^{+} cell count in the spleen. In non-immunized ApoE$^{-/-}$ mice *P. gingivalis* infection increased the number of CD3^{+} cells compared to uninfected ApoE$^{-/-}$ mice. In *P. gingivalis* infected ApoE$^{-/-}$ mice, the number of CD3^{+} cells was significantly decreased in immunized ApoE$^{-/-}$ mice as compared to non-immunized mice. *$p < 0.05$, ***$p < 0.001$, One-way ANOVA. NS indicates no significant differences.

Supplemental Figure 4. The ratio of spleen weight to body weight. Spleen weight was significantly increased in *P. gingivalis* infected / immunized ApoE$^{-/-}$ mice compared to the other groups. *$p < 0.05$, **$p < 0.01$ One-way ANOVA. NS indicates no significant differences.

Supplemental Figure 5. Collagen type III formation was diminished by immunization following *P. gingivalis* infection. Histological analysis of the total collagen content by picrosirius red staining visualized in bright field (**A-D**) and circularly polarized light (**E-H**). (**A and E**) group i: uninfected / non-immunized, (**B and F**) group ii:

uninfected / immunized, (**C and G**) group iii: *P. gingivalis* infected / non-immunized, and (**D and H**) group iv: *P. gingivalis* infected / immunized ApoE$^{-/-}$ mouse. Total collagen (**I**) and collagen type I / III ratio (**J**) was quantified. * $p < 0.05$, One-way ANOVA. NS indicates no significant differences.

Supplemental References

1. Thiele J., Kvasnicka H.M.Czieslick C., CD34+ progenitor cells in idiopathic (primary) myelofibrosis: a comparative quantification between spleen and bone marrow tissue, Ann Hematol, 2002, 81: 86-89.