

Online supplement for
Ogg1-dependent DNA repair regulates NLRP3 inflammasome and prevents atherosclerosis

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Supplementary methods.

Materials.

Antibodies: anti-Ogg1 (Proteintech group, Rosemont, Illinois), anti-cytochrome c and anti-GAPDH (Santa Cruz Biotechnologies, Dallas, Texas), and anti-8-OHdG (Bioss, Atlanta, Georgia). The FAM-FLICA kit was purchased from Immunochemistry Technologies and the TUNEL staining kit from Millipore. Secondary fluorescently labeled antibodies were from Molecular Probes (Invitrogen, Carlsbad, California) and the total RNA isolation kit was from Qiagen (Valencia, California). AICAR was purchased from Abcam (Cambridge, MA).

Cell culture.

Bone marrow derived macrophages (BMDM) were prepared as previously described¹.

Western blot analysis.

As described earlier², cells were lysed in lysis buffer with protease inhibitor cocktail (Roche). Cell lysates were rotated at 4°C for 0.5 h before the insoluble material was removed by centrifugation at 12,000 × *g* for 10 min. After normalization for equal protein concentration, cell lysates were resuspended in SDS sample buffer before separation by SDS-PAGE and transferred onto to PVDF membrane. Membranes were then incubated in blocking buffer and primary antibodies were applied in blocking buffer overnight at 4°C. Membranes were washed and incubated with a secondary antibody conjugated with horseradish peroxidase (GE Healthcare) for 2h at room temperature. After washing the membranes, a signal was detected by chemiluminescence using ECL Plus Western Blotting Detection kit (GE Healthcare Life Sciences, Piscataway, NJ) and a BioSpectrum UVP Imaging system (Bio-Rad, City, CA).

Plasma Lipid profiles.

Blood was obtained by retro-orbital puncture. Total cholesterol, high- and low density lipoprotein and triglyceride in the plasma were measured by colorimetric assay: Cholesterol E, L-Type Triglyceride M, L-Type LDL-C, HDL-Cholesterol E (Wako Diagnostics, Richmond, Virginia) per the manufacturer's instructions.

Glucose tolerance test.

Mice were fasted overnight (16 hours). Glucose dose was calculated based upon 2.5 g/kg body weight. Zero time point blood glucose was measured by glucometer with tail vein blood for each mouse. Injection volume i.p. was calculated using $BW(g) \times 10$ ul of 250mg/ml glucose solution. Blood glucose was measured at 30, 60, and 120 minutes after the glucose injection.

Insulin tolerance test

Mice were fasted for four hours. Insulin dose was calculated based upon 0.75 U/kg body weight. Zero time point blood glucose was measured by glucometer with tail vein blood for each mouse. Injection volume i.p. was calculated using $BW(g) \times 7.5$ ul of 0.1U/ml insulin solution. Blood glucose was measured at 30, 60, and 120 minutes after the glucose injection.

Creation of Bone-marrow chimeric mice.

8-week old recipient Ldlr KO mice were irradiated 9.5 Gy (Gammacell 40 Cs γ -irradiation) to eliminate endogenous BM stem cells and most of the BM-derived cells. Bone marrow from donor mice was harvested by flushing the femurs and tibias with PBS (pH 7.4). Single-cell suspensions were prepared by passing the cells through a 70 μ m cell strainer (BD, Breda, The Netherlands). Next, 2×10^6 donor BM cells were injected into the tail vein of recipient irradiated mice. The mice were kept in microisolator cages for 8 weeks to allow full BM reconstitution. After recovery, chimeric mice were placed on western diet for additional 12 wks. At the termination of each experiment, bone marrow was harvested and genotyping PCR analysis for the corresponding genes (Ogg1 or Nlrp3) was performed on BM derived macrophages, peritoneal macrophages, blood PBMCs (donor) ECs (recipient) to confirm chimerism.

Serum levels of chemokines and cytokines.

Serum concentrations of MCP-1, IL-6, IL-18, TNF- α and IL-12-P40 (eBiosciences, San Diego, California) were detected by ELISA according to the manufacturer's instructions.

Meso Scale Discovery (MSD).

IL-1 β in mouse plasma samples was measured using the U-PLEX Mouse IL-1 β Assay (Meso Scale Diagnostics, Rockville, Maryland) per the manufacturer's instructions. The samples were read and analyzed by MSD QuickPlex SQ120 instrumentation and Workbench 4.0 Software (Meso Scale Diagnostics, Rockville, Maryland).

miR-33 binding site identification.

The online target prediction programs www.targetscanhuman.com and www.microRNA.org were used to find potential targets for miR-33a and -b.

miRNA transfection.

Hela and immortalized BMDM B6-MCL cells were transfected with 20 nM mirVana miRNA mimic (miR-33a -3p and -5p) or with 30 nM mirVana miRNA inhibitors (Inh-miR-33a-3p and -5p) (Life technologies) utilizing XfectTM (Clontech). 48h after transfection, select cells were treated with 1mM AICAR for 2h (Abcam).

3'-UTR luciferase reporter assays.

HEK-293 cells were cotransfected with 0.75 μ g of OGG1 3'-UTR luciferase reporter and empty control vectors (abm) and 20 nM miR-33a-3p and -5p mimics or negative-control mimic using Xfect transfection reagent (Clontech). Luciferase activity was measured after 48h using the luciferase assay and was quantified as fold increase compared to the empty control vector transfected cells. Experiments were performed in duplicate and repeated at least three times.

Retroviral transduction

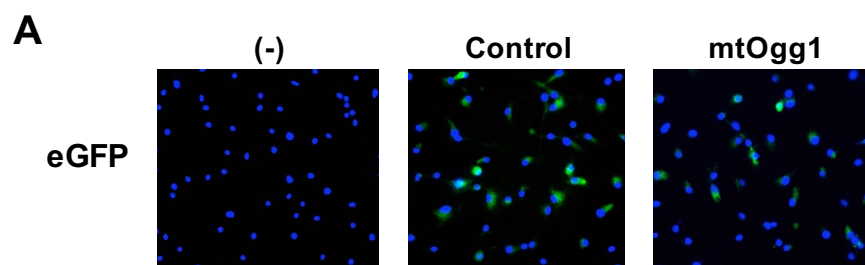
MIGR1-GFP and MIGR1-mtOGG1-GFP retroviruses were generated according to the manufacturer's instructions (Clontech Laboratories, Mountain View, California) and transduced into Ogg1 KO BMDM cells for 48h. The expression of eGFP were examined by fluorescent microscopy.

Human atherosclerotic lesion analysis

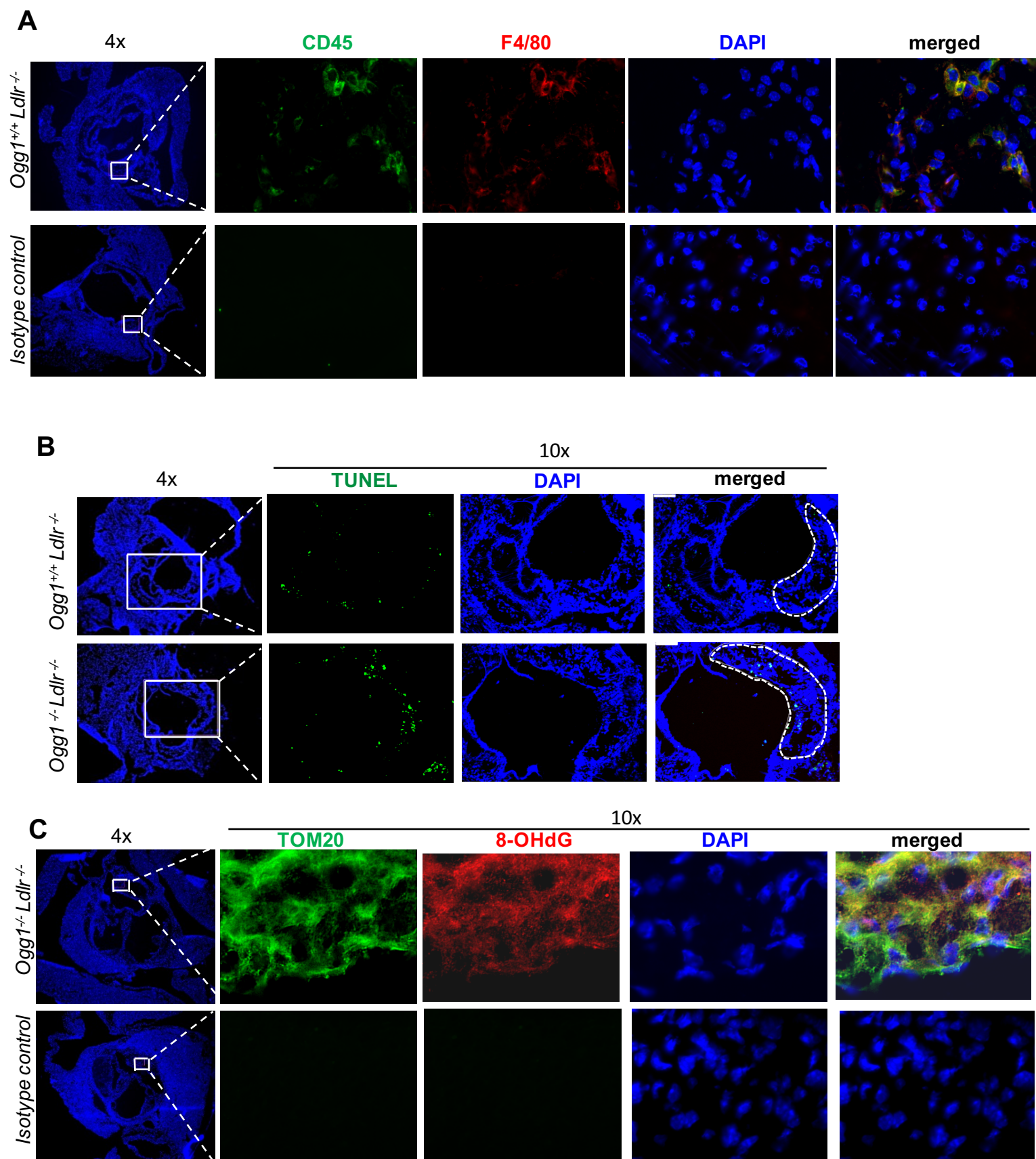
Human cases for study were identified by search of the pathology patient database at Cedars Sinai Medical Center (IRB# 00044050). The large-bore coronary arteries of explanted heart specimens (transplantation) and carotid artery specimens (endarterectomy) removed for atherosclerotic disease were identified. Reports and glass slides were reviewed, and the appropriate tissue in paraffin blocks were utilized for this study. The large-bore coronary arteries of explanted heart specimens of non-atherosclerotic resection specimens served as normal control tissue. The tissue within the paraffin blocks was homogenized with a TissueLyser II (Qiagen) and used for RNA isolated (Clontech). Total RNA was reverse transcribed using the QuantiTect kit (Qiagen) following the manufacturer's protocol. Quantitative real-time PCR was performed in triplicate using SYBR green mix (Takara, Clontech) using the following primers: 18S RNA FP: 5'-GGC CCT GTA ATT GGA ATG AGT 3' and 18S RP: 5' CCA AGA TCC AAC TAC GAG CTT 3'; OGG1 FP: 5'-CAC ACT GGA GTG GTG TAC TAG-3' and OGG1 RP: 5'-CCA GGG TAA CAT CTA GCT GGA A -3'. The mRNA level was normalized to 18S RNA as a house keeping gene.

References:

1. Shimada K, Crother TR, Karlin J, Dagvadorj J, Chiba N, Chen S, Ramanujan VK, Wolf AJ, Vergnes L, Ojcius DM, Rentsendorj A, Vargas M, Guerrero C, Wang Y, Fitzgerald KA, Underhill DM, Town T and Arditi M. Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity*. 2012;36:401-14.
2. Dagvadorj J, Shimada K, Chen S, Jones HD, Tumurkhuu G, Zhang W, Wawrowsky KA, Crother TR and Arditi M. Lipopolysaccharide Induces Alveolar Macrophage Necrosis via CD14 and the P2X7 Receptor Leading to Interleukin-1alpha Release. *Immunity*. 2015;42:640-53.

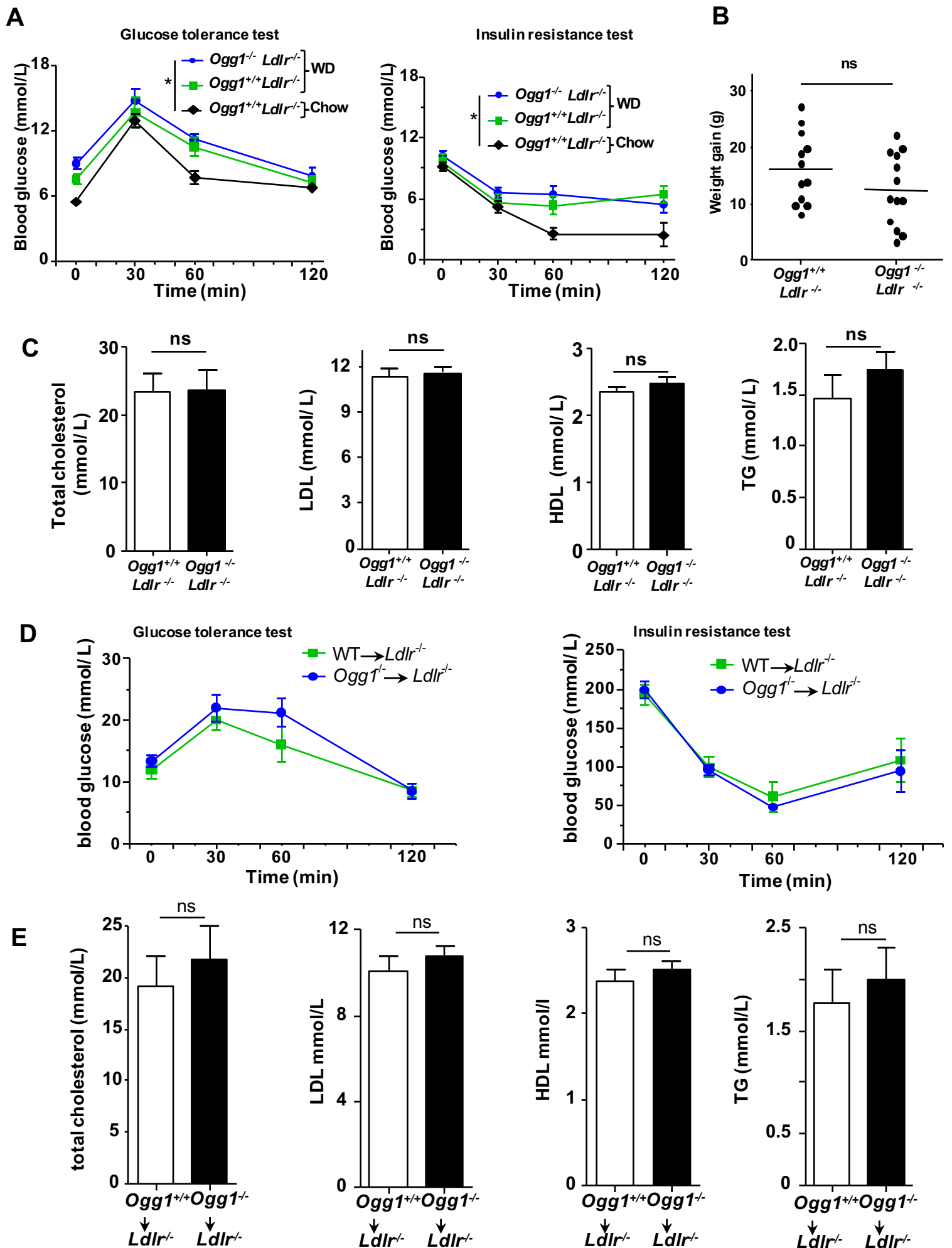


Supplemental Figure I. (A) Representative images of eGFP expression in retrovirus infected BMDMs. **Similar results were observed in 3 independent experiments.**



Supplemental Figure 2.

(A) F4/80 (marker of macrophage) is predominantly expressed in hematopoietic cells (CD45 positive) in aortic root plaques. Immunostaining for F4/80 (red), CD45 (green) and nuclei (blue). (B) Representative pictures of *in-situ* TUNEL staining (green) and nuclei (blue) for apoptosis. (C) Representative pictures of 8-OH-dG (red) and mitochondria (TOM20; green) in lesion.



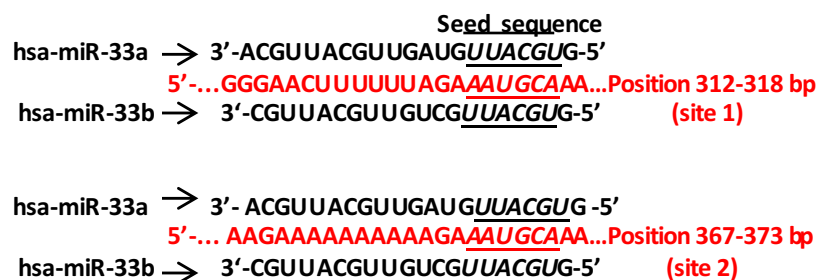
Supplemental Figure 3.

Supplemental Figure III.

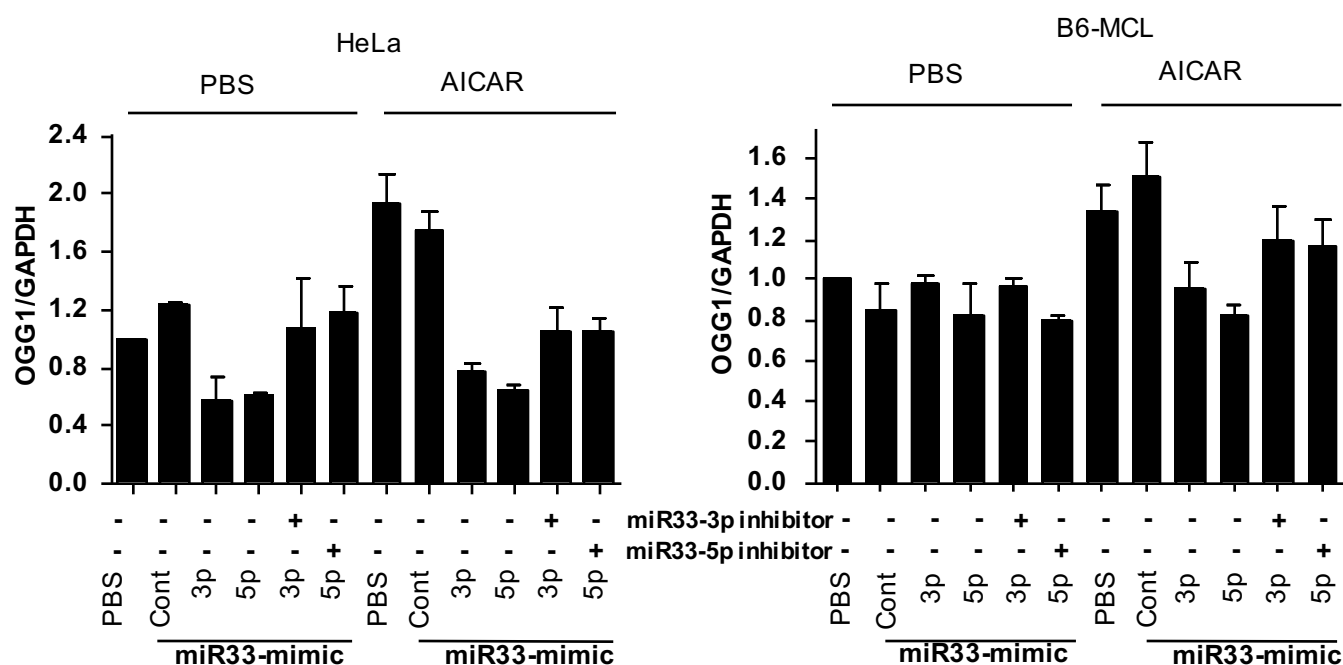
***Ogg1*^{-/-}*Ldlr*^{-/-} mice develop similar degree of glucose tolerance and insulin resistance as *Ldlr*^{-/-} mice.** *Ldlr*^{-/-} and *Ogg1*^{-/-}*Ldlr*^{-/-} mice were fat fed WD for 16 weeks (n=12). **(A)** Glucose tolerance and Insulin resistance test. **(B)** Body weight gain during WD. **(C)** Plasma lipid profile. Total cholesterol, HDL, LDL and TG concentration in plasma. **(D)** *Ogg1*^{-/-} BM chimeric mice on *Ldlr*^{-/-} develop glucose tolerance and insulin resistance similarly to control chimeric *Ldlr*^{-/-} mice. BM from WT or *Ogg1*^{-/-} mice transferred into irradiated *Ldlr*^{-/-} mice and after 8 week reconstitution, mice were fed a HFD for 12 weeks. Fasted glucose tolerance test (n=5). Insulin resistance (n=5). **(E)** Plasma lipid profile (n=5). **(A and D)** Significance was determined by calculating the area under the curve for each replicate (mice), and then groups were compared by One-Way ANOVA with Tukey's post-hoc test. **(B, C and E)** All data are means±SD. Significance was determined by Students T test. NS=not significant. **(C and E) The experiments performed in triplicates.**

A

Predicted miR-33 binding sites within the 3'-UTR of hOGG1 mRNA

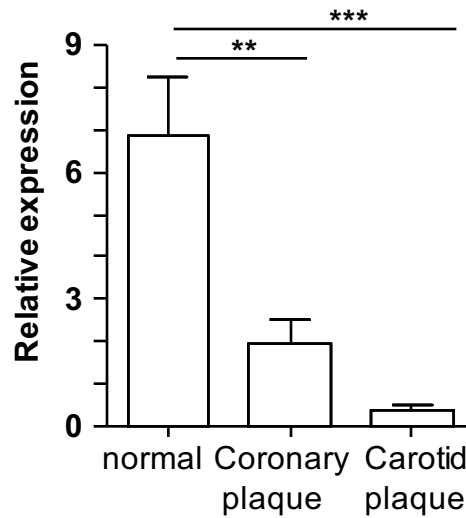


B



Supplemental Figure IV. (A) miR-33 specifically targets the 3' UTR of human OGG1. Annealing of 3'-UTR of OGG1 is complementary to miR-33a and -b. hsa indicates homo sapiens. **(B) Densitometric quantification of OGG1 protein amounts normalized by GAPDH amounts in HeLa (human) and B6-MCL (mouse) cells by Western blots shown in Figure 7C and D. The data is representative of 2-3 independent experiments.**

A



Supplemental Figure V. *OGG1* mRNA in human atherosclerotic plaques (A) The expression of *OGG1* mRNA in healthy arteries (Normal) (n=8) or coronary (n=8) and carotids (n=6) from patients with atherosclerosis (Plaque) from the CSMC. **The PCR was performed in triplicates.** Significance was determined using One-Way ANOVA with Tukey's post-hoc test. (** $P \leq 0.01$, *** $P \leq 0.001$).

Supplemental Figure V.