Supplemental Materials

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Supplemental Methods

Experimental animals

Animal study protocols were approved by the Animal Care Committee of Ewha Womans University. To generate *Prdx2*$^{-/-}$*ApoE*$^{-/-}$, Prdx2-deficient mice were crossed with *ApoE*-deficient mice. Both strains were *C57BL/6* congenic lines backcrossed more than 10 times with *C57BL/6J* mice. The atherogenic cholate-containing diet contained 0.15% cholesterol, 20% fat, and 0.05% sodium cholate (all w/w; Research Diets Inc., New Brunswick, NJ; C12348). Mice were euthanized and hearts and aortas perfused with phosphate-buffered saline (PBS) through the left ventricle. Hearts were embedded in OCT (Sakura, Tokyo, Japan) and frozen on dry ice. Aortas were dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and adventitial fat was removed. For *en face* analysis, aortas were split longitudinally, pinned onto flat, black silicone plates, and fixed in 10% (v/v) formaldehyde in PBS overnight. Fixed aortas were stained with oil red O for 4 h, washed with PBS briefly, and digitally photographed at a fixed magnification. Total aortic areas and lesion areas were calculated using AxioVision (Carl Zeiss, Jena, Germany). For analyzing aortic sinus plaque lesions and aortic arch lesions, cryosectioning was performed. Each section was stained with oil red O overnight and images were digitized. Plasma lipid levels were measured using an automatic blood chemical analyzer (Hitachi, Tokyo, Japan).

Bone marrow transplantation

ApoE$^{-/-}$ and *Prdx2*$^{-/-}$ *ApoE*$^{-/-}$ mice were euthanized using carbon dioxide, and the femurs and tibiae dissected. Sterile PBS was used to flush the marrow from each bone and then the marrow pooled.

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MACS beads (Miltenyi Biotec, Germany) conjugated with CD5^{+} Ab were used to deplete mature T cells from the bone marrow. Four-week-old *ApoE*$^{-/-}$ and *Prdx2*$^{-/-}$ *ApoE*$^{-/-}$ mice were lethally irradiated using gamma rays (10 Gy). Bone marrow cells (1×10^6) of *ApoE*$^{-/-}$ or *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ in 200 μl of sterile PBS were administered to each animal intravenously 24 h later. After transplantation, the mice were fed a normal chow diet for 4 weeks. Next, transplanted mice were placed on an atherogenic cholate-containing diet for 10 weeks prior to *en face* and aortic sinus assays. To ascertain engraftment, the Prdx2 wild-type and null alleles were amplified by PCR. Following 10 weeks of atherogenic cholate-containing diet, blood leukocyte genomic DNA was isolated from recipient mice using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Using the same method, blood leukocyte genomic DNA was isolated from the whole bodies of *ApoE*$^{-/-}$ and *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ mice. Genomic DNA from each mouse was PCR-amplified in duplicate for 30 cycles with specific primers for the Prdx2 wild-type or null alleles. Primer sequences used were 5'-TAGTCCAGGCC TTTCAGTA-3', 5'-AACTTCCTGACTAGGGGA GGAG-3', and 5'-GCTTTTGAAGGTGTGTGC CACC-3.

Infusion protocol for ebselen

Mice were anesthetized with avertin, and osmotic pumps (Durect, Cupertino, CA; Model 2004; 0.25μl per hour, 28 days) containing 168 μl of either 80% (v/v) DMSO or ebselen (Cayman Chemical, Ann Arbor, MI) were implanted subcutaneously. Ebselen was dissolved in 80% (v/v) DMSO and administered at a dose of 10 mg/kg/day. After 4 weeks, a second osmotic pump was implanted.

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Primary mouse aortic endothelial-cell isolation and culture

To isolate MAECs, aortas were placed on a Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, CA), and spreading MAECs in matrigel were treated with dispase (BD Biosciences). Released cells were replated into 12-well plates (BD Falcon, San Jose, CA). Harvested MAECs were cultured in EGM-2 BulletKit media (BioWhittaker, Walkersville, MD). Isolated MAECs were confirmed by TNFα-induced expression of adhesion molecules and uptake of Dil-Ac-LDL (Biomedical Technologies, Stoughton, MA; data not shown). All MAECs used in this study were between passages 3 and 4.

Aorta organ culture

Isolated aortas were longitudinally halved and cultured in a 24-well plate with 500 μl per well of complete Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY) containing 10% (v/v) fetal bovine serum, penicillin/streptomycin, and L-glutamine. Aortas were stimulated with 10 ng/ml recombinant TNFα (Millipore) for 12 h. Thirty minutes before TNFα stimulation, aortas were pre-treated with DMSO, 20 μmol/L ebselen (Cayman Chemical), or one of the following inhibitors (Calbiochem, San Diego, CA): BAY-11 (10 μmol/L), SB203580 (20 μmol/L), SP600125 (20 μmol/L), or PD98059 (20 μmol/L). After incubation (37°C, 5% [v/v] CO2, 12 h), aortas were transferred to 1.5-ml tubes, lysed with 40 μl of protein lysis buffer for preparation of lysates, and Western blotted for VCAM-1 and ICAM-1 expression. Each experiment was performed at least three times.

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***In vitro* and *ex vivo* adhesion assay**

$CD11b^+GFP^+$ cells were isolated from the bone marrow of green fluorescent protein (GFP) transgenic mice. Bone marrow cells were maintained in α-MEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum prior to use. For *in vitro* adhesion assay, MAECs were plated on 24-well plates at a density of 1×10^5 cells per well and cultured to confluence in EGM-2 BulletKit media. The cells were incubated with 10 ng/ml TNFα for 6 h, and 1×10^6 GFP^+CD11b^+ cells were added. After incubation for 30 min, unbound monocytes were removed by washing and the number of monocytes firmly bound to MAECs was counted in five consistent fields. For *ex vivo* adhesion assay, aortas were harvested from $ApoE^{-/-}$ or $Prdx2^{-/-}ApoE^{-/-}$ mice opened up longitudinally, and pinned onto sterile agar.

***In vitro* transmigration assay**

Migration of $CD11b^+$ monocytes was performed using Corning 3421 (Corning, Lowell, MA) in a 24-well plate fitted with a collagen-precoated polycarbonate filter with 5.0-μm pores according to the manufacturer's instructions. MAECs (5×10^4) were plated on inner wells and cells were stimulated with 10 ng/ml TNFα for 6 h. After stimulation, 1×10^5 $CD11b^+$ monocytes were loaded on MAECs. After incubation for 4 or 18 h, the number of cells that had migrated into the lower chamber was estimated using a cell counter. For analysis of transmigration rate, isolated MAECs from $Prdx2^{+/+}$ and $Prdx2^{-/-}$ mice on gelatin coated cover slides were activated by TNFα (10 ng/ml) for 6 h, and $CD11b^+$ monocytes were loaded for 10 or 30 min. Alexa Fluor 488 phalloidin was used to stain for F-actin to recognize the shape of endothelial cells, and nuclei were stained with 4',6-diamidino-2-phenylindole

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(DAPI). Images of transmigrating cells were randomly obtained by confocal microscopy. The transmigration rate of cells was analyzed by Z stack with AxioVision software and estimated as the percentage of transmigration depth of leukocytes over endothelial layer thickness.

En face hydrogen peroxide imaging

Isolated thoracic aortas from *Prdx2*$^{+/+}$ and *Prdx2*$^{-/-}$ mice were incubated in serum-free DMEM (without phenol red, Gibco) for 3 h, and stimulated with TNFα (10 ng/ml, with or without 30-min pre-treatment with ebselen) for the indicated times, washed with Hanks' Balanced Salt Solution, incubated with 10 μM CM-H_2DCFDA (Molecular Probes, Eugene, OR) in DMEM for 30 min, and mounted for confocal microscopy (Carl Zeiss; LSM 510 meta instrument). To observe H_2O_2 production in endothelial cells of the aorta, images were projected and auto-fluorescence from the elastic lamina removed using AxioVision software.

Quantitative real-time PCR

Total aortic RNA was isolated from the aortic arch of 20-week-old mice using Trizol reagent (GibcoBRL), and cDNA was synthesized with the Superscript III First-strand synthesis system (Invitrogen, Carlsbad, CA). Quantitative real-time RT-PCR was performed using a 7700 sequence detector (Applied Biosystems, Foster City, CA) and mouse VCAM-1, ICAM-1, E-selectin, and GAPDH primers and probes (Applied Biosystems, Assay-on-Demand™). The absolute number of gene copies was normalized using GAPDH and standardized using a sample standard curve.

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Reagents

For sandwich enzyme-linked immunosorbent assay, Duoset Ab pairs detecting MCP-1 were purchased from R&D Systems (Minneapolis, MN). Antibodies used included anti-ERK1/2, anti-phospho-(Thr202/Tyr204)-ERK1/2, anti-p38 MAPK, anti-phospho-(Thr180/Tyr182)-p38 MAPK, anti-SAPK/JNK, anti-phospho-(Thr183/Tyr185)-SAPK/JNK, anti-NF-κB p65, and anti-phospho-(Ser536) NF-κB p65 (Cell Signaling Technology, Danvers, MA). Anti-Prdx2 for Western blot was obtained from Ab Frontier, and anti-Prdx2 (sc-33572) for immunohistochemistry was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-c-Jun, anti-β-actin, GAPDH, and anti-VCAM-1 were purchased from Santa Cruz Biotechnology. To block VCAM-1 or ICAM-1, we used anti-mouse VCAM-1 Ab (R&D Systems, AF643) and anti-mouse ICAM-1 (R&D Systems, AF796).

Immunostaining

For *en face* confocal microscope imaging, isolated aortic arches and TA regions were permeabilized with 0.25% (v/v) Triton X-100 in PBS, washed, blocked for 1.5 h in 10% (v/v) chicken serum, and incubated with anti-VCAM-1 or ICAM-1 Ab (R&D Systems) in 10% (v/v) chicken serum (overnight, 4℃). After additional washing, aortas were incubated with Alexa Fluor-594 anti-goat IgG (Invitrogen) in PBS for 2 h at room temperature, and with DAPI (Sigma-Aldrich) in PBS for 20 min. Fluorescence was imaged using a confocal microscope (Carl Zeiss; LSM 510 Meta instrument). Rabbit anti-MCP-1, rabbit anti-TNFα (Santa Cruz Biotechnology), rat anti-CD4 (BD Biosciences), and rat anti-MOMA2 (AbD Serotec, Kidlington, UK) were the primary antibodies used for aortic

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sinus staining. Tissues were incubated with primary antibodies, incubated with biotinylated secondary antibodies, incubated with horseradish peroxidase-conjugated streptavidin, and visualized with DAB substrate (Vector Laboratories). Negative control tissues were stained with rabbit, rat, goat, and mouse IgG isotype control antibodies (Vector Laboratories and Santa Cruz Biotechnology). Nuclei were stained with hematoxylin or DAPI. Stained images were quantitatively analyzed using AxioVision (Carl Zeiss).

Statistical analysis

Results were analyzed using the Wilcoxon rank sum test for comparing two groups, or the Kruskal-Wallis test followed by Wilcoxon rank sum test for multiple comparisons. For matched experiments, the results were analyzed using the Wilcoxon signed rank test.

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Supplemental Table and Figures

Online Table I. Plasma total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol levels (mmol/L) of each group

Normal chow diet (20 weeks)	Female mice	
	ApoE $^{-/-}$ (n=7)	*Prdx2* $^{-/-}$ *ApoE* $^{-/-}$ (n=7)
Total cholesterol levels (mmol/L)	287.57 ± 56.13	267.57 ± 70.26
Triglyceride levels (mmol/L)	75.00 ± 18.74	85.43 ± 20.71
HDL cholesterol levels (mmol/L)	14.00 ± 2.51	12.00 ± 2.76
LDL cholesterol levels (mmol/L)	53.28 ± 9.10	50.57 ± 8.73

Normal chow diet (20 weeks)	Male mice	
	ApoE $^{-/-}$ (n=7)	*Prdx2* $^{-/-}$ *ApoE* $^{-/-}$ (n=7)
Total cholesterol levels (mmol/L)	333.29 ± 75.80	400.29 ± 77.44
Triglyceride levels (mmol/L)	95.14 ± 16.79	105.86 ± 27.40
HDL cholesterol levels (mmol/L)	24.71 ± 3.63	26.29 ± 3.98
LDL cholesterol levels (mmol/L)	48.43 ± 8.96	49.71 ± 9.69

Atherogenic cholate-containing diet (10 weeks)	Female mice	
	ApoE $^{-/-}$ (n=10)	*Prdx2* $^{-/-}$ *ApoE* $^{-/-}$ (n=10)
Total cholesterol levels (mmol/L)	1015.20 ± 175.96	915.60 ± 118.09
LDL cholesterol levels (mmol/L)	222.6 ± 46.49	210.6 ± 44.16

Atherogenic cholate-containing diet (10 weeks)	Male mice	
	ApoE $^{-/-}$ (n=4)	*Prdx2* $^{-/-}$ *ApoE* $^{-/-}$ (n=4)
Total cholesterol levels (mmol/L)	1248 ± 195.20	957.75 ± 251.12
LDL cholesterol levels (mmol/L)	243 ± 32.58	189 ± 49.77

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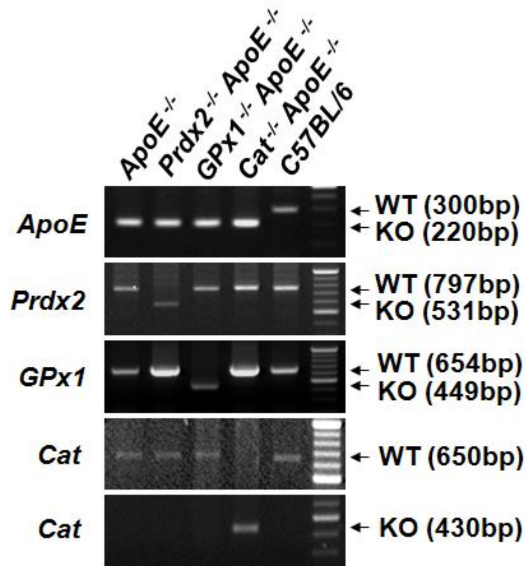

Online Figure I. Generation of ***Prdx2$^{-/-}$ApoE$^{-/-}$*****,** ***GPx1$^{-/-}$ApoE$^{-/-}$, and Cat$^{-/-}$ApoE$^{-/-}$*** **mice.** Prdx2, GPx1, or Cat deficient mice were crossed with ApoE-deficient mice, respectively.

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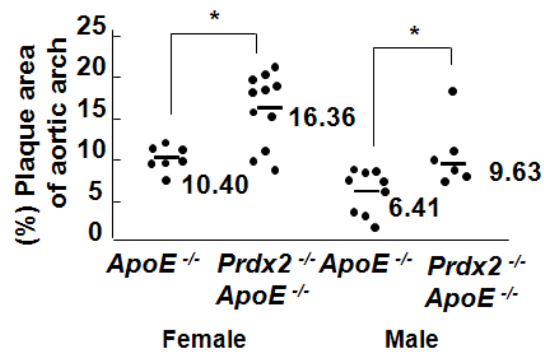

Online Figure II. Increased plaque formation in *Prdx2*$^{-/-}$ *ApoE*$^{-/-}$ mice. Representative oil red O staining of aortas from 20-week-old *ApoE*$^{-/-}$ or *Prdx2*$^{-/-}$ *ApoE*$^{-/-}$ mice. Quantitative data (means ± SD; n = 6–11, *$P < 0.05$) in the graph represent plaque percentage in aortic arches.

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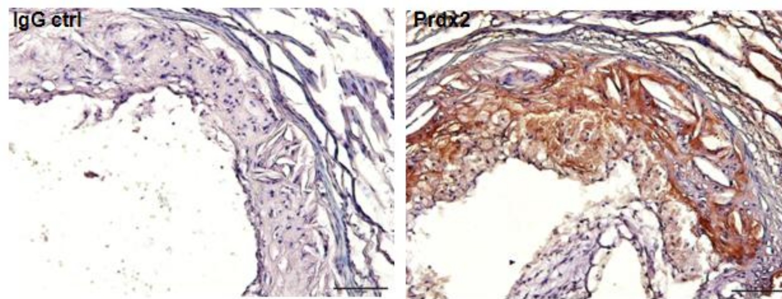

Online Figure III. Prdx2 expression in the plaque. Representative immunostaining for Prdx2 on aortic sinus sections from *ApoE*$^{-/-}$ mice fed an atherogenic cholate-containing diet for 10 weeks. Bars, 100 μm.

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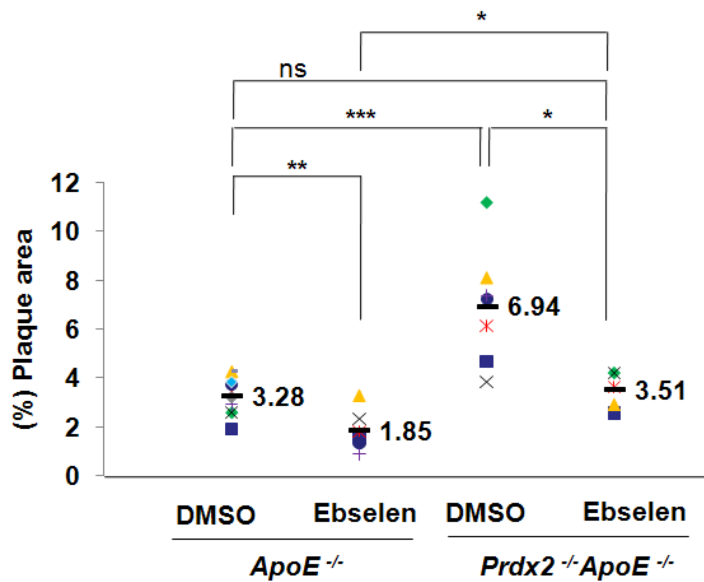

Online Figure IV. Administration of ebselen reduced plaque formation in *Prdx2$^{-/-}$ApoE$^{-/-}$* mice. *ApoE$^{-/-}$*, *Prdx2$^{-/-}$ApoE$^{-/-}$* mice infused with DMSO or ebselen (10 mg/kg/day), and fed an atherogenic cholate-containing diet for 8 weeks. Measurement of plaque areas in *ApoE$^{-/-}$* or *Prdx2$^{-/-}$ ApoE$^{-/-}$* mice infused with DMSO or ebselen (10 mg/kg/day). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control group.

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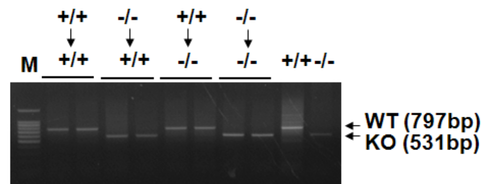
A



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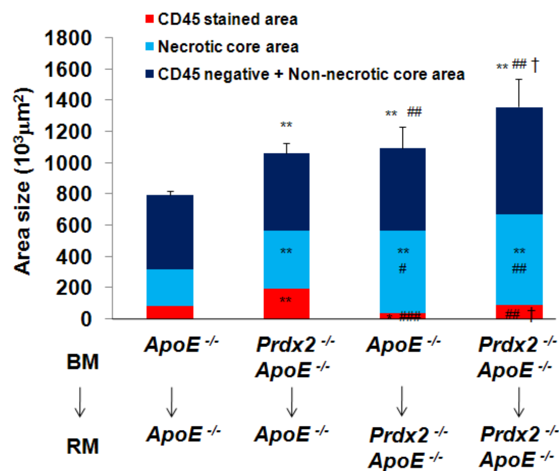

Online Figure V. Identification of chimerism and CD45$^+$ cell accumulation in bone marrow-transplanted mice. A, PCR identification of chimerism in blood cells from bone marrow-transplanted mice. +/+ indicates the *ApoE*$^{-/-}$ genotype and -/- indicates the *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ genotype. **B**, Prdx2 deficient bone marrow-transplanted mice had increased accumulation of CD45$^+$ cells in plaques,

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and necrotic core size in aortic sinuses of *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ recipient mice increased over 2.3-fold (*ApoE*$^{-/-}$ BM) and 1.5-fold (*Prdx2*$^{-/-}$*ApoE*$^{-/-}$ BM) compared to *ApoE*$^{-/-}$ recipient mice (mean ± SD; n = 5–8). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. *ApoE*$^{-/-}$ recipients with *ApoE*$^{-/-}$ BM, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. *ApoE*$^{-/-}$ recipients with *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ BM, $^{\dagger}P < 0.05$ vs. *Prdx2*$^{-/-}$*ApoE*$^{-/-}$ recipients with *ApoE*$^{-/-}$ BM.

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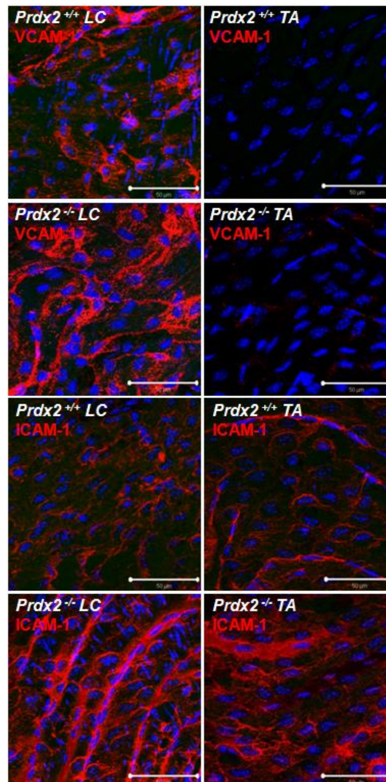

Online Figure VI. Increasing expression of VCAM-1 and ICAM-1 by Prdx2 deficiency. *En face* immunofluorescence staining of VCAM-1 and ICAM-1 expression in the lesser curvature (LC) and thoracic aorta (TA) regions from *Prdx2*$^{+/+}$ and *Prdx2*$^{-/-}$ mice (n = 5) fed the atherogenic cholate-containing diet for 2 weeks. Bars, 50 μm.

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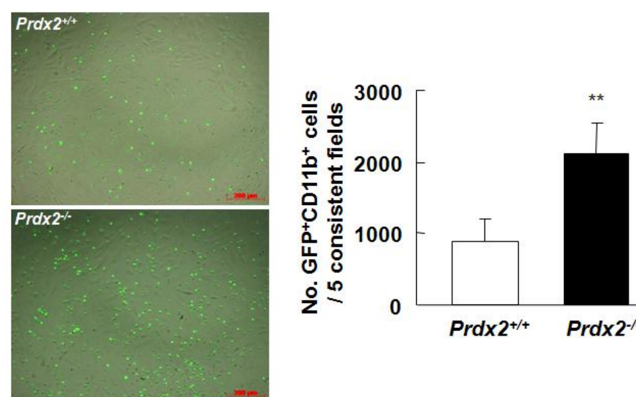

Online Figure VII. Increased monocyte adhesion in *Prdx2*$^{-/-}$ MAEC. Representative *in vitro* binding assay of GFP^{+}CD11b^{+} cells on *Prdx2*$^{+/+}$ or *Prdx2*$^{-/-}$ MAECs stimulated with TNFα (10 ng/ml) for 12 h. The graph represents number of bound cells. Bars, 200 μm. $^{**}P < 0.01$ compared to *Prdx2*$^{+/+}$ MAECs.

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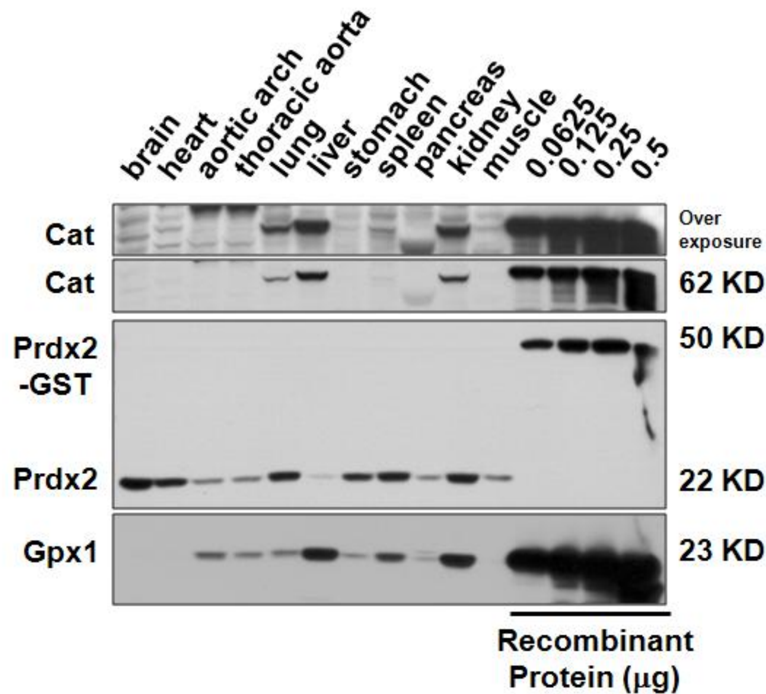

Online Figure VIII. Semi-quantification of Prdx2, GPx1, and Cat in tissues of *C57BL/6* mice. Western blots were performed on protein lysates from brain, heart, aortic arch, thoracic aorta, lung, liver, stomach, spleen, pancreas, kidney, and muscle of *C57BL/6* mouse for recombinant Prdx2-GST, GPx1, and Cat proteins. Lysates (40 μg) and recombinant proteins (indicated amounts) were loaded onto SDS-PAGE gels. The density of mPrdx2-GST recombinant protein was evaluated approximately 2.5-fold more vividly compared to the density of GPx1 or Cat recombinant protein; because the size of mPrdx2-GST recombinant protein grows larger than natural size of Prdx2. Levels were developed on the same membrane for each recombinant protein.

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