Materials and Methods

Animals and Diets

C57BL/6J (000664) and *Ldlr*$^{-/-}$ (002381) mice on the C57BL/6J background were purchased from Jackson Laboratory. mCAT transgenic mice were generated as described previously[1, 2], and were backcrossed >10 times onto the C57BL/6J background. For the atherosclerosis studies, mCAT transgenic and age-matched and gender-matched littermates were used as donors. Six weeks after bone marrow transplantation (at 14 weeks of age), *Ldlr*$^{-/-}$ male mice remained fed chow diet. Starting at the age of 36 weeks, mice were placed on the Western-type diet (WD; TD88137; Harlan Teklad) for 16 weeks. For the young animals, six weeks after bone marrow transplantation (14 weeks old), the recipient animals were placed on WD for 16 weeks.

Bone Marrow Transplantation (BMT)

8-week-old male Ldlr-/- mice were lethally irradiated using an X-ray source (Precision X-RAD 320 Biological Irradiator) at a dose of 1000 rad 4–6 h before transplantation. Bone marrow cells were collected from the femurs and tibias of donor wild-type and mCAT transgenic mice by flushing with sterile medium as described previously[1]. All animal procedures used in this study followed Columbia University's institutional guidelines.

Atherosclerotic Lesion Analysis

For morphometric lesion analysis, sections were stained with Harris hematoxylin and eosin (H&E). Total lesion area and necrotic area were quantified as previously described[3]. Briefly, paraffin-embedded specimens were sectioned to 6μm thick, deparaffinized with xylene, and rehydrated in decreasing concentrations of ethanol. Slides were then stained with H&E, and images were viewed and captured with a Nikon Labophot 2 microscope equipped with an Olympus DP25 color digital camera attached to a computerized imaging system with Image-Pro-Plus software (version 3.0; Mediacybernetics). Total intimal lesion area and necrotic areas per cross section were quantified by taking the average of 6 sections spaced 30 μm apart beginning at the base of the aortic root. Intimal lesion area is defined as the areas from internal elastic lamina to the lumen. Necrotic area were defined as the acellular and anuclear region in the intima of aortic root lesions. These regions were H&E staining free. Necrotic core was defined as a necrotic area larger than 3,000-μm^2. Using this method, a 97% agreement in the percent necrotic area was calculated between 2 independent observers. For immunofluorescence staining, the sections were blocked by PBS containing 10% goat serum, and then incubated overnight at 4°C with anti-Mac-3 (1:200; BD Clone M3/84), anti-8-hydroxydeoxyguanosine (1:200; anti-8-OHdG; AB5830; EMD Millipore), MPO-biotinylated (1:30; R&D, BAF3667), Ly6G (1:200, Biolegend, Clone 1A8), Histone H3 Citrulline (1:250; Abcam ab5103). The sections were then incubated with Alexa488-labeled, Alexa594-labeled, streptavidin-conjugated Alexa488-labeled antibodies (Life Technologies). Sections were counterstained with DAPI to identify nuclei before mounting, and visualized by fluorescence microscopy.

Measurement of NET release in Atherosclerotic Lesions and Cultured Neutrophils

Paraffin-embedded specimens were sectioned and stained with MPO (1:30; R&D, BAF3667) and Histone H3 Citrulline (1:250; Abcam ab5103). We detected NETs as large amorphous extracellular structures which were the areas of MPO positive signal co-localized with Histone H3 Citrulline signal in the intimal areas of the lesions.[4] Mouse neutrophils were isolated from

25-week-old male C57BL/6J mice peripheral blood using mouse neutrophil isolation kit (Miltenyi Biotec, 130-097-658) according to the manufacture's instruction. Briefly, ~300ul whole blood was lysed with RBC lysis buffer (BD, 555899) and WBCs were washed with cold autoMACS Running Buffer (Miltenyi Biotec,130-091-221). Cells were then stained with mouse neutrophil Biotin-antibody cocktail antibody followed by anti-biotin microbeads. Beads-labeled WBCs were resuspended in autoMACS Running Buffer and loaded onto MS column, and the pass through liquid was collected. Neutrophils were spun down, resuspended in full culture medium containing RPMI (Corning, 10-041-CV), 0.5% BSA, 1% penicillin/streptomycin/glutamine solution (GIBCO), and then plated in Poly-D-lysine-coated plates for 30 minutes @ 37°C with 5% CO_2 ($4x10^4$/well of 48-well plate). Cells recovered from this procedure were validated by flow cytometry, and >95% of cells were Ly6C positive. Cells were then pretreated with mitoTEMPO (Enzo, ALX-430-150-M005) for 30 minutes and 200ng/ml PMA (Sigma, P8139) for additional 4 hours. At the end of incubation, cells were washed with cold PBS and fixed/permeabilized with ice-cold acetone (Sigma, 650501), and stained with anti-8-OHdG (Abcam, ab62623) and anti-histone H3 Citrullin antibodies and DAPI. Cells were visualized by fluorescence microscopy. NET were visualized in 16 random images in each well, quadruplet for each animal. Results were quantified as the proportion of NET releasing cells among total DAPI+ cells and as NET-covered areas from each neutrophil using Image J. NETs were seen as string-like structure extending from the cell body that were positive for DNA and histone, which we quantified as extracellular material that was double-positive for DNA ($DAPI^+$), citrullinated-H3 ($Cit\text{-}H3^+$) and MPO^5.

Measurement of 8-OHDG in Lesional Myeloid Cells

Evidence of mitoOS in lesional macrophages was obtained by assaying oxidative damage of non-nuclear (mitochondrial) DNA, as described previously[1]. Specifically, paraffin-embedded specimens were sectioned and were stained sequentially with anti-8-OHDG and anti-Mac-3 primary or anti-Ly6G antibodies, Alexa488-labeled anti-rabbit and Alexa594-labeled anti-rat antibodies, and DAPI, which was used to measure the total number of cells and to identify nuclei. Sections were then imaged by fluorescence microscopy. Data were quantified as percentage of total $Mac3^+$ cells and as total number of $Mac3^+$ cells showing 8-OHDG staining that did not overlap with DAPI, that is, as an indicator of exposure of mitochondrial DNA to oxidative stress.

Measurement of Mitochondrial Superoxide and Cytosolic ROS in Cultured Neutrophils

Cultured neutrophils were incubated with warm culture media containing 5μM mitochondrial superoxide indicator mitoSOX (Life Technology, M36008) for 30 minutes. Cells were then rinsed with warm media and followed by the indicated treatment. At the end of incubation, mitoSOX mean fluorescence intensity (MFI) was determined by flow cytometry. To measure cytosolic ROS, cells were first treated with indicated stimuli and then stained with 0.6μM cytosolic ROS indicator CellROX (Life Technology, C10491) for 20 minutes. CellROX MFI was also measured by flow cytometry.

Plasma Glucose, Cholesterol and Triglyceride Measurements

Fasting blood glucose levels were measured using ONETOUCH Ultra strips after 12 h of fasting. Total plasma cholesterol and triglyceride were measured using commercially available kits (Wako Pure Chemical Industries).

Measurement of Mitochondrial Mass in Peripheral Leukocyte

Briefly, blood was lysed by cold RBC lysis buffer, and total peripheral leukocytes were stained with 100nM MitotrackerGreen (MTG) at 37°C with 5% CO_2 for 15 min. Cells were then stained with antibodies against CD115, Gr-1, CD45 on ice for 15 min before flow cytometry analysis, using FSC/SSC and $CD45^+$ gating. The $CD115^+Gr\text{-}1^+$ population was defined as $Ly6C^{high}$ monocytes; $CD115^+Gr\text{-}1^-$population as $Ly6C^{low}$ monocytes; and $CD115^-Gr\text{-}1^+$ as neutrophils. Mitochondrial mass was determined by the mean fluorescent intensity (MFI) of MTG[6, 7].

Measurement of neutrophil trans-well migration in vitro.

Mouse neutrophils isolated from peripheral blood from 58-week-old (old) or 16-week-old (young) male mice were resuspended in culture medium (RPMI + 0.5% BSA) at the concentration of 125,000 cells/ml. Cell suspension was injected into the inserts of FluoroBlok™ HTS 96 Well Multiwell Permeable Support System with 3.0µm High Density PET Membrane (Corning), 100 µl/well. 200µl medium containing 10µM Formyl-Met-Leu-Phe (Sigma Aldrich, F3506) was injected into receiver plate. Cells were allowed to migrate for 2 hr at 37°C with 5% CO_2, and then stained with 10µg/ml Hoechst (ThermoFisher Scientific, H3570) for 10 min. Total number of cells that migrated toward the bottom of receiver plate was quantified by counting the number of $Hoechst^+$ cell in each well.

Statistics

Values are given as means ± S.E.M. unless otherwise noted, with n number for each experiment listed in the figure legends. Comparison of mean values between two groups was usually evaluated by a Student t-test. When the data did not fit a normal distribution, the MannWhitney U rank-sum test was used. Comparison of mean values between more than two groups was evaluated by one-way ANOVA followed by Bonferroni post hoc test. For all statistical methods, a P value less than 0.05 was considered significant.

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