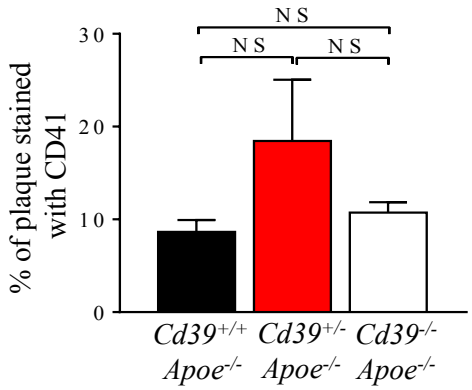
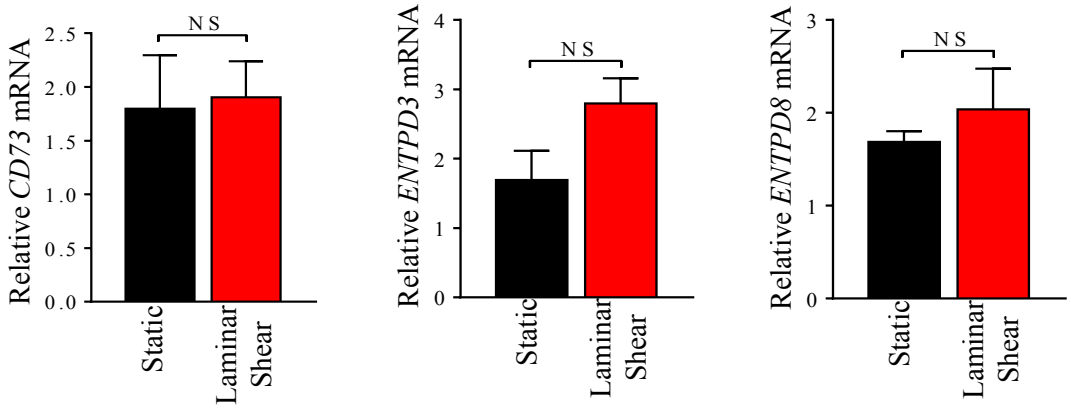
	Heart rate (beats/min)	Systolic pressure (mmHg)	Total Cholesterol (mg/mL)	Triglycerides (mg/mL)
***Cd39*$^{+/+}$ *Apoe*$^{-/-}$**	543±51	100±20	999±190	91.5±17.5
***Cd39*$^{+/-}$ *Apoe*$^{-/-}$**	563±42	92±6	1238±245	91.5±12.3
***Cd39*$^{-/-}$ *Apoe*$^{-/-}$**	561±64	95±10	1410±350	90.3±14.4

Supplemental Table 1. CD39 expression does not alter hemodynamic or plasma lipids in *Apoe*$^{-/-}$ mice. Blood pressure, heart rate and plasma lipids were similar in *Cd39*$^{+/+}$ *Apoe*$^{-/-}$, *Cd39*$^{+/-}$ *Apoe*$^{-/-}$, and *Cd39*$^{-/-}$ *Apoe*$^{-/-}$ mice (*n*=5-9 per group). Student's *t* test. *P*, no statistical difference.



Supplemental Figure 1. Platelet deposition is unaltered in atherosclerotic plaques *Cd39* expression-altered hyperlipidemic mice. Platelet deposition was examined by staining for the platelet surface marker CD41 in atherosclerotic plaques of *Cd39*$^{+/+}$ *Apoe*$^{-/-}$, *Cd39*$^{+/-}$ *Apoe*$^{-/-}$, and *Cd39*$^{-/-}$ *Apoe*$^{-/-}$ mice, and quantified (n=5-7 per group). Student's t test. NS, no statistical significance.



Supplemental Figure 2. Laminar shear stress does not alter gene expression of the human endothelial cell surface nucleotidases *Cd73* (also known as *NT5E*), *ENTPD3* or *ENTPD8*. HUVEC were exposed to laminar shear stress (15 dynes/cm^2) for 48 hours did not affect transcript levels of non-CD39 ecto-nucleotidases normally expressed at the endothelial cell surface (n=3-5 per group). Student's t test. NS, no statistical significance.

Methods:

All animal experiments were conducted in accordance with the NIH guidelines for use of live animals and this protocol was approved by the Institutional Animal Care and Use Committees at the University of Michigan.

Tissue staining for CD39 expression

Apolipoprotein E-deficient mice (*Apoe*$^{-/-}$) (Jackson Laboratories, Bay Harbor, Maine) fed a standard chow diet were sacrificed at 32 weeks of age. The left ventricle was cannulated and the mice were perfused with saline (Gibco, Carlsbad, California) and 10% buffered formalin (Fisher Scientific, Pittsburgh, Pennsylvania). The aortic arch was carefully dissected free of excess adventitial tissue and embedded in paraffin. Paraffin sections (5.0 μm) were made and antigen retrieved using citrate buffer (Vector Labs, Burlingame, California). Slides were stained for CD39 with a rat-anti-mouse CD39 monoclonal antibody generated by the University of Michigan Hybridoma Core as previously described (55) and verified for detection specificity to CD39 by ELISA as previously described (41). Slides were developed using a VECTASTAIN ABC-Peroxidase kit (Vector Labs, Burlingame, California) and TSA Tetramethylrhodamine Tyramide Reagent Pack (Perkin-Elmer, Wellesley, Massachusetts). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images were acquired with a 40x objective with a 1.5x magnifying lens (0.107171 um/pixel) using MetaMorph v7.0r3 software (Molecular Devices, Sunnyvale, California) on an Eclipse TE2000-E microscope (Nikon Instruments, Inc., Melville, New York).

Cerium-chloride staining of ATPase activity

Twenty-six week-old, *Apoe*$^{-/-}$ mice were euthanized prior to cerium-chloride staining to measure liberation of inorganic phosphate following ATP application as previously described (51). The left ventricle was perfused with ice-cold 0.05 M Na-cacodylate buffer, pH 7.2 (Electron Microscopy Sciences, Hatfield, Pennsylvania). Aortas were fixed for 5 minutes *in situ* with a mixture of ice-cold 2%

paraformaldehyde, 0.5% glutaraldehyde (Electron Microscopy Sciences), 0.25 mM sucrose, and 2 mM $MgCl_2$ in 0.05 M cacodylate buffer (pH 7.2). The aortic arch was dissected free and post fixed for 10 additional minutes in fixation buffer. Tissue was embedded in 7% low melting point agarose prior to sectioning with a Vibratome (70 μm). Tissue sections were washed overnight in cacodylate buffer and then rinsed briefly in Tris-maleate buffer (pH 7.2). Slides were incubated for 20 min at 37°C in reaction buffer: Tris-maleate (0.1 M, pH 7.2), ATP (2 mM), $MgCl_2$ (2 mM), KCl (20 mM), $CeCl_3$ (2 mM), 2 mM levamisole, 100 mM ouabain, and 50 μM a,b-methylene-ADP, and 0.0001% Triton X-100. Samples were rinsed in Tris-maleate buffer (pH 6.0) and cacodylate buffer (pH 7.2) prior to post-fixation with 1% OsO_4 (Electron Microscopy Sciences, Hatfield, Pennsylvania). After rinses in distilled water, tissue slices were dehydrated in graded ethanol, processed through propylene oxide, and embedded in Epon (Fluka, Buchs, Switzerland). Ultrathin 40 nm sections were cut using a Reichert Ultracut-E (Leica Microsystems Inc., Bannockburn, Illinios) and imaged with a Philips CM-100 transmission electron microscope.

Generation of $Cd39^{-/-}Apoe^{-/-}$ and $Cd39^{+/-}Apoe^{-/-}$ Mice

To investigate the *in vivo* effects of *Cd39* gene-deletion on atherogenesis, mice null in the enzymatically active apyrase-conserved regions 2-4 of the *Cd39* gene (26) were bred with $Apoe^{-/-}$ mice on a C57BL/6 background (Jackson Laboratories). $Cd39^{-/-}$ mice were previously backcrossed a minimum of 6 generations onto the C57BL/6 background. Animals were backbred for 6 generations to produce *Apoe*-deficient-*Cd39*-deficient ($Cd39^{-/-}$ $Apoe^{-/-}$) and *Apoe*-deficient-*Cd39* hemizygous ($Cd39^{+/-}$ $Apoe^{-/-}$) littermates. At 4 weeks of age male littermates were placed on a diet containing 42% calories from fat (Harlan Teklad, Madison, Wisconsin). At 20 weeks of age, animals were euthanized after a 12-hour fast.

Measurement of atherosclerotic lesion size of $Apoe^{-/-}$ mice

Aortas from $Cd39^{+/+}$ $Apoe^{-/-}$, $Cd39^{+/-}$ $Apoe^{-/-}$, and $Cd39^{-/-}$ $Apoe^{-/-}$mice that had been fed a 42% calories from fat diet for 16 weeks were collected and stained with oil red O as previously described (56).

Images were taken using a DP12 Olympus Microscope Digital Camera and the percent surface areas occupied by oil red O−stained lesions were determined using Image-Pro Plus v.4.5 analysis software (Media Cybernetics, Rockville, Maryland).

Measurement of CD39 expression

For immunoblotting, equal amounts of purified membrane and whole cell protein fractions were resolved with a non-reducing Bis-Tris gel in LDS buffer (Invitrogen, Carlsbad, California). Gels were transferred onto PVDF (Invitrogen) and the membranes were probed with antibodies. Murine CD39 was probed with a rat-anti-mouse CD39 monoclonal antibody generated by the University of Michigan Hybridoma Core as previously described (55). Human CD39 was recognized with a mouse anti-human CD39 monoclonal antibody (Ab30422; Abcam, Cambridge, Massachusetts) or rabbit anti-mouse CD39 polyclonal antibody (sc33558; Santa Cruz, Dallas, Texas). HRP-conjugated anti-rat and anti-mouse antibodies were used, respectively, as secondary antibodies (Sigma, St. Louis, Missouri) and developed by ECL Detection Reagent (GE Amersham, Piscataway, New Jersey). Data was normalized to β-actin using HRP-conjugated anti-mouse or anti-human β-actin antibodies (Sigma, St. Louis, Missouri).

Cellular membrane preparations from tissue homogenates

WT, *Cd39*$^{+/-}$, *Cd39*$^{-/-}$ mice were euthanized and perfused with ice-cold saline via a right ventricle cannula (Gibco, Langley, Oklahoma). To obtain cell membrane preparations from a rich vascular source, murine lungs were excised and placed in 2 mL of ice-cold, hypotonic lysis buffer (50 mM sucrose, 10 mM Hepes, pH 7.4, with 1 μg/ml aprotinin, 1 μg leupeptin, 1 mM PMSF). The lungs were minced using a Tissuemizer tissue homogenizer (Fisher Scientific). Following the addition of 132 μl of 65% sucrose [(w/w) in 10 mM Hepes, pH 7.4], 4 μl of 0.5 M $MgCl_2$ into 2 ml of hypotonic lysis buffer, the homogenate was subjected to two 20-minute spins at 2,000 g at 4°C to pellet nuclei, mitochondria, and unlysed cells. Crude membranes were pelleted from the resulting supernatant by centrifugation at 100,000

g for 30 min at 4°C, washed quickly in 2 ml of hypotonic lysis buffer, and resuspended in the same buffer. The membranes were flash frozen in aliquots and stored at -80°C. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, California).

Whole-cell protein isolation

Cells were washed with PBS (Invitrogen), scraped with a rubber policeman, and suspended in ice-cold RIPA buffer with protease inhibitor (Roche, Branchburg, New Jersey). Cells were homogenized by 10 strokes through a sterile, 30 gauge 1 ml insulin syringe. Lysed cells were centrifuged for 10 min at 13,000 *g* at 4°C. Supernatants were aliquoted and flash frozen at -80°C. Concentrations were determined using a colorimetric protein assay (Bio-Rad, Hercules, California).

Measurement of CD39 function

Apyrase activity of the purified mouse membrane fraction was assayed with a Malachite Green Phosphate Assay Kit (BioAssay Systems, Hayward, California) per the manufacturer's instructions and analyzed using a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, California).

Radio-thin layer chromatographic (TLC) was used to assess CD39 enzymatic function of live HUVEC (41, 52). Human umbilical vein endothelial cells (1×10^5) were treated with static or laminar shear stress. Equal number of cells in Medium 199 (Invitrogen) from each treatment were mixed with 1.0 mM [8-14C]ATP (ARC, Saint Louis, Missouri) in the presence of 286 μM AOPCP (Sigma, St. Louis, Missouri), a CD73 inhibitor to arrest conversion of AMP to adenosine and incubated at 37°C for 40 min. Reactions were stopped using 8M formic acid. The reaction mixture was spotted onto silica gel TLC plates (Invitrogen). A ladder of [8-14C]ATP (ARC), [8-14C]ADP (Perkin Elmer, Waltham, MA), and [8-14C]AMP (Amersham, Piscataway, New Jersey) was used. Nucleotides were separated by thin layer chromatography with a solvent mixture of isobutyl alcohol, isoamyl alcohol, 2-ethoxyethanol, ammonia, and water (9:6:18:9:15) (52). Separation was allowed to occur for 6 hours before plates were allowed to dry. Silica plates were exposed to a phosphorimaging screen (Eastman Kodak Co., Rochester, New

York), and then analyzed using a Typhoon Trio Variable Mode Imager (GE Healthcare, Livonia, Michigan).

Preparation and staining of cryosections

Frozen sections of the aortic root sinus were prepared as previously described (57) . Aortic sinus sections (5.0 μM) were briefly fixed with acetone. Platelet remnants were identified by staining for CD41/GP11b (clone MWReg30; AbD Serotec, Raleigh, North Carolina) at a dilution of 1:300. Macrophages were stained with CD68 (clone FA-11; AbD Serotec, Raleigh, North Carolina) at a dilution of 1:300 and detected with an AlexaFluor 488 pre-conjugated secondary antibody (A-11006; Molecular Probes). Primary antibody was detected using a TSA™ Tetramethylrhodamine System (Perkin-Elmer, Wellesley, Massachusetts). Images were acquired using MetaMorph v7.0r3 software on an Eclipse TE2000-E microscope (Nikon Instruments, Melville, New York).

Measurement of plasma cytokines and lipids

Plasma was isolated from whole blood was drawn into citrated syringes via the inferior *vena cava* from mice that had been fasted for 12 hours overnight. Plasma was analyzed per the manufacturer's protocol using a Mouse CCL5/RANTES Quantikine ELISA kit (R&D Systems, Minneapolis, Minnesota), Mouse sP-Selectin/CD62P Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota), and an Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, Oregon), using a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, California).

Whole blood aggregometry

Heparinized murine blood was drawn from the retro-orbital plexus of *Cd39*$^{+/+}$, *Cd39*$^{+/-}$, and *Cd39*$^{-/-}$ mice on either WT or *Apoe*$^{-/-}$ backgrounds, at 12 weeks of age. Whole blood was allowed to sit for 25 minutes prior to being diluted 4-fold with a 1:1 mixture of normal saline and lactated ringer's solution prewarmed to 37°C. The blood/electrolyte mixture was allowed to equilibrate for 5 minutes in the cuvette

of the Chrono-log 560CA whole blood aggregometry machine (Chrono-log, Havertown, Pennsylvania). The whole blood samples were then treated with either 0.75 μM, 5.0 μM, or 10 μM ADP, and aggregation was recorded using the 810 Aggrolink Interface.

Cell culture

Primary peritoneal macrophages were obtained and cultured as previously described (31). RAW cells (murine macrophage RAW 264.7 cells. ATCC, Manassas, Virginia) were cultured as previously described (53). Human umbilical cords were obtained with permission from the University of Michigan Institutional Review Board. Primary HUVEC were isolated from umbilical cords and cultured as previously described (41). HUVEC and human aortic endothelial cells (HAEC) (PCS-100-011, ATCC, Manassas, Virginia) were grown on 0.2% gelatin-A coated plates in endothelial basal medium with endothelial growth medium supplements (Lonza, Allendale, New Jersey). HUVEC were used up to passage 4 and HAEC were used at passage 8-11.

Overexpression of CD39 in RAW 264.7 macrophages.

RAW macrophages were transfected with murine *Cd39* or pCDNA3.1 vector (Invitrogen) as a control as previously described (31) using SuperFect Transfection Reagent (QIAGEN) according to manufacturer protocol. RAW cells were grown in RPMI 1640 (Invitrogen) containing 10% FCS (Invitrogen) in 37°C, humidified atmosphere containing 5% CO_2.

LDL uptake assay

Cells were incubated for 4h with DiI-AcLDL (10 μg/mL) or DiI-OxLDL (10 μg/mL) (Biomedical Technologies, Stoughton, Massachusetts) in RPMI-1640 at 37°C. DiI-AcLDL and DiI-OxLDL uptake were analyzed with fluorescence microscopy and with a fluorescent plate reader (Molecular Devices, Sunnyvale, California). DiI-AcLDL and DiI-OxLDL uptake are represented as a percentage with the vehicle-treated control indicated as 100%. Photomicrographs were taken using a

Nikon Eclipse TE2000-E microscope. In experiments with purinergic receptor inhibitors, cells stimulated with TNP-ATP, suramin or ox-ATP as previously described (31).

Quantitative reverse transcription-PCR

Quantitative reverse transcription-PCR (qRT-PCR) was used to quantify RNA levels. After treatment, cells were washed twice in PBS, and then total RNA was isolated using RNeasy kits (Qiagen, Valencia, California). cDNA was made using cDNA synthesis kits (Applied Biosystems, Grand Island, New York). Real-time quantitative PCR was carried out using the 7000 detection system (Applied Biosystems) with universal mastermix and Taqman primers (Applied Biosystems). All data were normalized to β2 microglobulin or 18s rRNA in shear stress experiments.

Partial carotid ligation

Partial carotid ligation was performed on 8-10 week old male C57Bl/6 (Jackson Laboratories, Bay Harbor, Maine) as previously described (16, 21). Briefly, mice were anesthetized and 3 of 4 caudal branches of the left carotid artery (left external carotid, internal carotid, and occipital artery) were ligated with 6-0 silk suture, leaving the superior thyroid artery intact. At 48 hours, carotid arterial flow patterns were examined *in vivo* using Doppler ultrasonography with a Vevo2100 40 MHz transducer (VisualSonics, Toronto, Canada). Mice were then euthanized and saline-perfused via the left ventricle. Right and left common carotid arteries were harvested, and endothelial RNA was isolated as previously described (16, 21). Gene expression was analyzed using qRT-PCR.

KLF2 gene silencing studies

Short hairpin RNA (shRNA) was generated by inserting a hairpin loop into small-interfering RNA (siRNA) sequences for *KLF2* 5'-GCACCGACGACGACCUCAAUU-3' (50) and a nonspecific control 5'-AUGAACGUGAAUUGCUCAAUU-3' (19). Lentivirus was produced at the University of

Michigan Vector Core. Briefly, lentivirus packaging vectors psPAX2 (35 μg) and pC1-VSVG (35 μg) were co-transfected with 70 μg of pLentilox3.7 (ATCC), and Lenti-Scramble-VSVG (shScramble) or Lenti-*KLF2*-VSVG (shKLF2) shRNA proviral plasmid into 293T cells using standard PEI precipitation methods. Supernatants were collected and pooled after 72 hours, filtered, pelleted and resuspended at ~$1x10^7$ TU/mL in DMEM (Invitrogen). HUVEC or HAEC were transduced with shKLF2 or shScramble for 48 hours prior to treatment with static or laminar shear stress. Bone marrow derived macrophages were transfected with siRNA (Invitrogen) against *KLF2* (siKLF2) or a scramble sequence (siScramble) using oligofectamine (Invitrogen) according to manufacturer's instructions.

In vitro shear stress studies

Cells were exposed to static shear conditions or arterial levels of laminar shear stress using a cone-and-plate shear apparatus, as modified for a 60 mm tissue culture dish (Corning) (54). Cells were grown to confluent monolayers and exposed to LS at 15 dynes/cm^2 for 48 hours. This level of shear stress is consistent with physiologic conditions and is widely used in *in vitro* shear experiments.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was carried out using a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling, Danvers, Massachusetts) according to manufacturer instructions. Briefly, HUVEC were treated with static or arterial levels of laminar shear stress for 48 hours prior to harvest. Native protein-DNA complexes were cross-linked with 1% formaldehyde for 15 min. Equal aliquots of isolated chromatin were subjected to immunoprecipitation with rabbit polyclonal anti-human KLF2 antibody (bs-2772R, Bioss, Woburn, Massachusetts) or with negative control rabbit immunoglobulin G (Cell Signaling). DNA was isolated and used as a template for the PCR to amplify the *CD39* promoter sequence containing the KLF2 consensus binding site closest to the transcriptional start site (TSS) using qRT-PCR in three separate experiments and performed in triplicate with Brilliant SYBR green mix. The primers used were a 5'- CTTGCAGCTGAGATGACTTTTT-3'and 5'–

TCTCTCCCTTACTCCCTCCCTCTT-3'. As a specificity control, non-KLF2 binding sites identified by *in silico* analysis were amplified from the same templates using the following primers: 5′-TGTGTGTTCACCTCACATG-3' and 5'-GAAAGACAATGAGGGCTTA-3'. Data were presented as fold change over DNA input.

Data analysis

Values are expressed as mean ± standard error of the mean (S.E.M.), with the number of experiments performed provided in the figure legends. For experiments in which two variables were compared, unpaired Student's *t* tests were used with Welch's correction. For experiments in which more than two variables were compared, one-way ANOVA was used, with Tukey's post hoc analysis to test for significant differences. Data were considered significantly different when *P* was less than 0.05.

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