Materials and Methods

Chemicals and Antibodies

All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Recombinant human IL-35 cytokine was purchased from Enzo (#ALX-522-140-C010; Farmingdale, NY); and recombinant mouse IL-35 was generously provided by Prof. D. Pascual (University of Florida, Gainesville). LPC (16:0) was purchased from Avanti Polar Lipids, Inc (#855675P; Alabaster, Alabama). For mtROS measurement, MitoSOX Red Mitochondrial Superoxide Indicator (#M36008; Life technologies, Carlsbad, CA) and mito-TEMPO-H Spin Probe (#ALX-430-171-M005; Enzo) were used. IRDye 700 AP-1 oligonucleotides were purchased from LI-COR (#829-07925; Lincoln, Nebraska) for detecting the nuclear translocated transcription factor bindings to the gene promoter of interest. Mitochondrial ROS inhibitor, MitoTEMPO, was purchased from Enzo (#ALX-430-150-M005). Anti-ICAM-1 antibody was purchased from Cell Signaling (#4915; Danvers, MA).

Animals and Human Samples

All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) Guidelines and Authorization for the use of Laboratory Animals and were approved by the IACUC of Temple University Lewis Katz School of Medicine, which confirmed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All mice used were on a C57BL/6 background. ApoE mutant mice, commonly known as $ApoE^{-/-}$ mice (strain name: B6.129P2-Apoetm1Unc/J), and

wild-type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Male mice were used in the ELISA experiments, while both males and female mice were used in the atherosclerosis quantification experiments. Both WT and ApoE$^{-/-}$ mice were weaned at 4 weeks of age. For high fat diet feeding, they were given 0.2% (w/w) cholesterol and 21.2% (w/w) fat high fat diet (TD.88137, Harlan, Hayward, CA). In accordance with a protocol approved by the Institutional Review Board at Temple University, plasma samples of de-identified patients and healthy controls were obtained from Bioreclamation IVT (East Meadow, NY), which confirmed the Declaration of Helsinki and preparation of informed consent forms.

Microarray Analysis

RNAs of the aorta from five male WT and ApoE$^{-/-}$ mice that were fed a Western diet for 3 weeks starting from 8 week-old were isolated with the RNeasy Kit (Qiagen). RNA quantity was determined by the NanoDrop ND-2000 (Thermo Scientific). RNA samples were sent to the Fox Chase Cancer Center Genomic Facility. The RNA integrity was determined by the RNA 28S/18S ratio using the Agilent 2100 Bioanalyzer (Agilent Technologies). Samples were then labeled and hybridized to the Affymetrix Genechip Mouse Gene 2.0ST Arrays, following the manufacturer's instructions. Scanned microarray images were analyzed using the Affymetrix Gene Expression Console with Robust Multi-array Average normalization algorithm.

Cell Culture

Human aortic endothelial cells (HAECs) (Lonza, CC2535; Walkersville, MD) were cultured in medium M199 (Hyclone laboratories, Logan, UT) supplemented with 15% fetal bovine serum (FBS; HyClone); endothelial cell growth supplement (ECGS, 50µg /mL; BD Biosciences, San Jose, CA); heparin (50µg/mL); and 1% penicillin, streptomycin, and amphotericin (PSA; Invitrogen, Carlsbad, CA). HAECs were grown on 0.2% gelatin-coated flasks, plates, or dishes and experiments were performed at passage 9.

Fluorescence Activated Cell Sorting (FACS)

After staining with MitoSOX (5µM), HAECs were incubated at 37^{o}C for 10 minutes (min) and washed with PBS twice afterwards. After LPC treatment, cells were washed once with ice-cold PBS and Trypsin-EDTA was added to detach cells. Trypsinization was terminated by adding FACS buffer (2% FBS in PBS); and cells were collected by centrifugation. After re-suspension in 0.2ml FACS buffer, samples were subjected to flow cytometry analysis, where fluorescence emissions were measured at FL2 channel by FACS Calibur machine (BD).

Electron Spin Resonance (ESR)

After LPC treatment, HAECs were washed with PBS twice and collected by cell scraper. HAECs were then centrifuged and collected in 100µl of deoxygenated, pH 7.35 Krebs HEPES buffer (KHB) (99mM NaCl, 4.69mM KCl, 25mM $NaHCO_3$, 1.03mM KH_2PO_4, D-glucose 5.6mM, Na-HEPES 20mM, $CaCl_2$(x2H_2O) 2.5mM, $MgSO_4$ 1.2mM). Cells were then vortexed briefly and aliquoted into 10µl suspension in Eppendorf tubes. To each aliquot of cells, 80 µl of modified KHB buffer (plus 25 µM Deferoxamine and 5 µM DETC)

and 10µl of MitoTEMPO-H buffer (10mM) were added to measure mtROS. The remaining cells were centrifuged and the cell pellets were frozen for protein concentration determination. MtROS were quantified by normalizing MitoTEMPO-H signal with protein concentrations.

Site-Specific Histone Acetylation Screening

Residue-specific histone acetylation was quantified by multiplexed mass spectrometry using published methods[1, 2]. Briefly, a Waters Acquity H-class UPLC (Milford, MA) coupled to a Thermo TSQ Quantum Access (Waltham, MA) triple quadrupole (QqQ) mass spectrometer was used to quantify site-specific acetylated H3 peptides. After histone extraction and purification by the Histone Purification Mini Kit (Active Motif), the extracted histones were subjected to propionylation and trypsin digestion. The digested samples were then injected to an Acquity BEH C18 column (2.1×50 mm; particle size 1.7 µm) with 0.2% formic acid (FA) aqueous solution (solution A) and 0.2% FA in acetonitrile (solution B). Peptides were eluted over 11 min at 0.6 mL/min and 60°C, and the gradient were programmed from 95% solution A and 5% solution B and down to 80% solution A and 20% solution B in 11 min. Selected reaction monitoring (SRM) were used to monitor the elution of the acetylated and propionylated H3 peptides. The resolved peaks were integrated using Xcalibur software (version 2.1, Thermo); and the relative quantitative analysis was used to determine the acetylation fraction on individual lysine residues.

Genome-wide Analysis of H3K14ac Binding Positions

To generate genome-wide maps of H3K14ac in HAEC, we performed chromatin (CH) immunoprecipitation (IP) followed by next-generation sequencing (CHIP-Seq) using previously published method[3]. In brief, after chromatin was isolated and cross-linked, the DNA in the chromatin was sheared to an average length of 300-500 base pairs (bp). Genomic DNA regions of interest were isolated by performing IP using specific antibody against H3K14ac (Active Motif, cat.# 39599). Crosslinks were reversed afterwards; and CHIP DNA was purified by phenol-chloroform extraction and ethanol precipitation. Quantitative PCR reactions were carried out in triplicate on specific house-keeping genes before next-generation sequencing (Illumina) was performed. Illumina sequencing libraries were prepared from the CHIP and Input DNAs (without IP) by the standard consecutive enzymatic steps of end-polishing, dA-addition, and adaptor ligation. After a final PCR amplification step, the resulting DNA libraries were quantified and sequenced on NexSeq 500. Sequences (75 nucleotide (nt) reads, single end) were aligned to the human genome (hg19) using the BWA algorithm (software package for mapping low-divergent sequences against a large reference genome, such as the human genome, default settings). Duplicate reads were removed and only uniquely mapped reads (mapping quality >= 25) were used for further analysis. Alignments were extended *in silico* at their 3'-ends to a length of 200 bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nt bins along the genome. The resulting histograms (genomic "signal maps") were generated. H3K14Ac enriched regions were identified using the spatial clustering for identification of CHIP-enriched regions[4] (SICER algorithm, threshold FDR 1E-10, gap size 600 bp). Using a cutoff of FDR 1E-20, significant changed-regions between the control sample and the LPC-treated sample

were further identified. Signal maps and peak locations were used as input data to acquire detailed information on sample comparison, peak metrics, peak locations and gene annotations.

RNA-Seq

Total RNA are extracted from samples, then mRNA and non-coding RNAs are enriched by removing rRNA from the total RNA with kit. By using the fragmentation buffer, the mRNAs are fragmented into short fragments (about 200~500nt), then the first-strand cDNA is synthesized by random hexamer-primer using the fragments as templates, and dTTP is substituted by dUTP during the synthesis of the second strand. Short fragments are purified and resolved with elution buffer for end reparation and single nucleotide A (adenine) addition. After that, the short fragments are connected with adapters, then the second strand is degraded finally using UNG(Uracil-N-Glycosylase) [2]. After agarose gel electrophoresis, the suitable fragments are selected for the PCR amplification as templates. During the quality control steps, Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System are used in quantification and qualification of the sample library. At last, the library is sequenced using Illumina HiSeq4000 using PE100 strategy.

Primary sequencing data that produced by Illumina Hiseq4000 called as raw reads, are filtered into clean reads by remove adaptor contained and low quality reads by BGI (Shenzhen, China) in-house software. Reference annotation based assembly method is used to reconstruct the transcripts by Tophat (v2.0.10) + Cufflinks (v2.1.1), while

background noise is reduced by using FPKM (Fragments Per Kilobase Million) and coverage threshold.

Western Blot Analysis

Protein extracts were collected from HAECs. Protein concentrations were determined by the bicinchoninic acid (BCA) assay with BSA standards. Proteins were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% BSA in Tris buffered saline containing 0.01% Tween 20 [TBST, 50mM Tris (pH 7.5), 150mM NaCl, and 0.1% Tween 20 (v/v)]. Membranes were incubated with primary antibodies overnight at 4°C. Membranes were then washed extensively with TBST and incubated with the appropriate horseradish peroxidase-labeled secondary antibodies for 1 hour at room temperature. Afterward, membranes were incubated with enhanced chemiluminescence (ECL) substrate for horseradish peroxidase (Pierce/Thermo, Rockford, IL) and the ECL intensity was detected by X-ray film exposure in a dark room. The X-ray films were developed by the SRX-101A medical film processor. The expression levels of proteins as indicated by the ECL intensity were measured with ImageJ software (NIH, Bethesda, MD, USA).

Electrophoretic Mobility Shift Assay

Transcription factor interactions with DNA response elements were assessed using electrophoretic mobility shift assay (EMSA). AP-1 consensus oligonucleotide end-labeled with IR700 was purchased from LI-COR (Lincoln, Nebraska). The sequence of the AP-1 probe is 5'-CGCTTGATGACTCAGCCGGAA-3'. EMSA was carried out using

an Odyssey Infrared EMSA kit (LI-COR) according to the manufacturer's instructions. 2 μg of nuclear extracts were added to each binding reaction. The probe and nuclear proteins were incubated for 20 min at room temperature and DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide gel afterwards. Images of gel were then obtained in an Odyssey scanner (LI-COR).

Atherosclerotic Lesion Analysis

Mouse hearts were harvested, weighed, and fixed overnight with 4% paraformaldehyde (PFA). Fixed tissues were then kept in 20% (v/v) sucrose (Sigma) embedded with optimal cutting temperature compound (OCT) (Fisher), and quickly frozen on dry ice. Mouse aortas were transferred to mini-centrifuge tubes containing PBS and stored at 4 °C. Serial cross sections of the aortic root were collected on slides; 8-μm cryostat sections were taken from the level where the 3 aortic valves first appeared to the level where the aortic valves disappeared. A total of 80 sections were collected on 10 slides. Sections of the aortic sinus were stained with Oil Red O and alum hematoxylin. Briefly, fixed sections were rinsed with 60% isopropanol and stained with freshly prepared Oil Red O working solution (0.3% Oil Red O in 60% isopropanol) for 18 min. Followed with another rinse with 60% isopropanol, the sections were then stained with alum hematoxylin and washed with distilled water. The stained sections were then mounted in aqueous mounting medium and stored in room temperature until imaging. Meanwhile, the aortas were used for *en face* staining. After fully removing all adipose and connective tissue, the aortas were stained with Sudan IV (5 mg/ml in 70% isopropyl alcohol) (Sigma) for 40 min at 37 °C. After this, the aortas were incubated in 70% isopropyl alcohol for 5

min. Next, a longitudinal cut was made to expose the intimal surface of the vessel and pinned open on a black silicone tray for imaging.

Images of the aortic root were captured with an AxioCam camera mounted to an Axioskop 2 microscope, whereas images of the aorta were captured with the same camera mounted to a Stemi 2000-C microscope (Carl Zeiss). Atherosclerotic lesion area within the aortic sinus was defined as the area stained with Oil red O and measured with ImageJ (National Institutes of Health). The absolute lesion area was measured, and the average value of sections on each slide was presented. Meanwhile, the lesion area in the whole aorta was defined as the red area stained with Sudan IV. Since male and female mice showed similar atherosclerotic lesion reduction after IL-35 treatment, the data from both genders were analyzed together.

Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) throughout the manuscript. For comparisons between two groups, two-tailed Student *t* test was used for evaluation of statistical significance or, when the data were not normally distributed, a nonparametric Mann-Whitney U test was used. For comparisons across multiple groups, one-way ANOVA with Bonferroni post-test adjustment was used or, when the data were not normally distributed, the data were analyzed using one-way ANOVA with the Kruskal-Wallis test, followed by pairwise comparison using the Dunn test. For linear regression tests, simple linear regression analyses were performed using GraphPad Prism to determine coefficient of determination and p value. Data shown are representative of two to three independent experiments. NS, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

References

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