Chemicals and reagents

RPMI-1640 (Cat. AL162S), HiEndoXL Endothelial growth medium (Cat. AL517), EnVzyme Easy (Cat. TCL137), bovine serum albumin (BSA; Cat. TC348), Dulbecco's phosphate buffer saline (PBS; Cat. TS1006), ethanol (Cat. MB106), gelatin (Cat. TC041), Hoechst 33342 (Cat. TC266), Oil red O stain (Cat. TC256) and antibiotic antimycotic solution (Cat. A002) were purchased from Himedia Laboratories, India. Oxidized LDL (Cat. L34357), Opti-MEM reduced serum media (Cat. 31985070), Fetal bovine serum (FBS; Cat. 10270-106), Dulbecco's minimal essential medium (DMEM; Cat. AL007S), lipofectamine 3000 reagent (Cat. L3000008), MitoTracker Red CMXRos (Cat. M7512), JC-1 (Cat. T3168), mouse monoclonal β-actin antibody (Cat. MA1-744), Alexa Fluor-488 anti-rabbit secondary antibody (Cat. A-11008), Alexa Fluor-568 anti-mouse secondary antibody (Cat. A-11004), Trizol reagent (Cat. 15596018) and Power Up SyBr Green master mix (Cat. A25741) were purchased from Thermo Fisher Scientific, USA. Protease inhibitor cocktail (PIC; Cat. P8340), phorbol 12-myristate 13-acetate (PMA; Cat. P8139), Fluoroshield with DAPI (Cat. F6057), Direct red 80 (Cat. 365548), Griess' reagent (Cat. 03553) and hematoxylin (Cat. MHS32), eosin (Cat. 109844) were obtained from Sigma Aldrich, USA. RNA-later stabilizing solution (Cat. AM7021) was purchased from Ambion Inc. (USA). Human HSP60 ELISA kit (Cat. ELH-HSP60) was purchased from RayBiotech, USA. Rabbit monoclonal HSP60 antibody (Cat. 12165), rabbit polyclonal β-actin antibody (Cat. 4970) and HRP linked anti-rabbit secondary antibody (Cat. 7074) was purchased from Cell Signaling Technologies, USA. BioRad Protein Assay Dye reagent concentrate (Cat. 5000006), Polyvinylidene fluoride (PVDF) membrane (Cat. 1620177), Precision Plus protein ladder (Cat.), iScript cDNA synthesis kit (Cat. 1708891) and Clarity

Western ECL blotting substrate (Cat. 1705061) were purchased from Bio-Rad Laboratories, USA. 3,3'-diaminobenzidine (DAB; Cat. K3468), mouse monoclonal CD68 antibody (Cat. M0718) and HRP-linked anti-mouse secondary antibody (Cat. P0447) were purchased from Dako, Agilent, USA. High Speed Plasmid mini kit (Cat. PD100) was obtained from Geneaid Biotech Ltd., Taiwan. Methanol (Cat. 96446), dimethyl sulfoxide (DMSO; Cat. 24075), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Cat. 58945), chloroform (Cat. 96712) and isopropanol (Cat. 38445) were purchased from Sisco Research Laboratory Pvt. Ltd. Mumbai, India. All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany).

Animal maintenance and ethical statement

The experimental protocol (MSU-Z/IAEC/2/09-2017) was approved by the Institutional Animal Ethical Committee (IAEC), Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara and experiments were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). C57BL/6J male mice each weighing 20-22g and aged 4-6 weeks were obtained from Advanced Center for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai, India and maintained at CPCSEA approved animal house (827/GO/Re/S/04/CPCSEA) of Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara. Animals were housed as 3-5 per cage and fed ad libitum with a standard laboratory chow and water in rooms maintained in 12 h light/12 h dark cycles (24 h LD cycles) with the temperature at 23°C to 25°C, humidity between 50%–70%. After a 10 days acclimatization period, they were randomly divided into experimental groups.

Blood and Tissue collection

At the end of the experimental protocol, mice were fasted overnight and blood was collected via retro-orbital sinus under mild isoflurane anaesthesia. Blood was allowed to clot at room temperature (RT) for 30 min and serum was isolated by centrifuging clotted blood at 3000 rpm at 4°C for 10 min. Serum thus obtained, was stored at -20°C till further used for analysis. After collecting blood, mice were euthanized and thoracic aorta were excised. For histochemical analysis, tissue were fixed in 4% PFA, dehydrated in a graded series of ethanol and embedded in paraffin using standard protocol. For immunoblotting, collected tissue were directly snap frozen in liquid nitrogen whereas for gene expression studies, tissues were collected in RNA later and then, snap frozen in liquid nitrogen. The frozen tissues were stored at -80°C until further use.

Serum lipid analysis

Serum isolated from blood was subjected to analysis of triglycerides (TG), total cholesterol (TC), very low density lipoprotein (VLDL), low density lipoprotein-cholesterol (LDL-Chol), high density lipoprotein-cholesterol (HDL-Chol) and LDL-Chol/HDL-Chol ratio using commercially available kits (Reckon Diagnostic kits, Vadodara, Gujarat, India).

Histology and morphometric analysis

5 µm serial sections of paraffin embedded thoracic aorta were cut using microtome and stained with hematoxylin–eosin (HXE) as described by Andres-Manzano et al. (2015). The sections were observed and photographed using Leica DMRB microscope (Leica Microsystems, Germany). The morphometric measurements were obtained using ImageJ software (NIH, Bethesda, USA) by an investigator blinded to the experimental grouping. Internal elastin lamella (IEL) and external elastin lamella (EEL) were manually marked

and their lengths were measured to obtain internal (lumen) perimeter and external perimeter of the thoracic aorta, respectively. The cross-sectional area was assumed to be circular in *in vivo* conditions so that both the perimeters represent the internal (i.e luminal) and external circumference. The internal and external diameters (D_i and D_e, respectively) were calculated from the respective circumferences, and the intima-media thickness (IMT) was calculated as:

$$\text{IMT} = \frac{\text{De}-\text{Di}}{2}$$

Also, the lumen area was calculated as:

$$\text{Lumen area} = \pi\,(D_i/2)^2$$

Where, D_e= external diameter, D_i= internal diameter.

Elastin autofluorescence

The autofluorescence of elastin lamellae was observed in HXE stained sections of thoracic aorta and images were captured using FLoid Cell Imaging Station (Thermo Fisher Scientific, USA) as the protocol described by Sawada and Daugherty (2018). Elastin fragmentation/break, defined as the discontinuity of an elastin fiber with boundaries at both sides clearly visible, were counted manually.

Collagen staining

Paraffin-embedded sections of thoracic aorta (5 µm) were de-paraffinized with xylene and rehydrated by immersion in a graded series of ethanol. The sections were the stained with 0.1% direct red 80 in saturated aqueous solution of picric acid for 1 h at RT and observed under Leica DMRB microscope. Images were captured using Canon power shot S70 digital camera and collagen content was measured relative to vascular area using ImageJ. Also,

the same sections were observed for fluorescence of collagen and elastin using FLoid Cell Imaging Station and images were captured. Simultaneous quantification of elastin and collagen content was carried out from these images using ImageJ as described by Borges et al. (2005) and Vogel et al. (2015). Using these values, collagen-to-elastin ratio was calculated as a measure of arterial stiffness.

Immunohistochemistry

Paraffin embedded sections (5 µm) were de-paraffinized in xylene, re-hydrated in graded series of ethanol and washed with phosphate buffered saline (PBS). Antigens were retrieved by heating the sections in sodium citrate buffer at 95°C for 20 min that was followed by masking of endogenous peroxidases with 3% hydrogen peroxide for 20 min in dark. Subsequently, the sections were blocked in 1% FBS for 30 min at RT and incubated overnight with mouse monoclonal CD68 antibody (Dako, Agilent, USA) and rabbit monoclonal HSP60 antibody (Cell Signaling Technology, USA) at a dilution of 1:100 and 1:200 respectively, at 4°C in humidified chamber. After washing with PBS, sections were incubated with HRP-conjugated anti-mouse secondary antibody and HRP conjugated anti-rabbit secondary antibody, respectively for 1 h at RT. Sections were washed thoroughly with PBS and DAB substrate was added followed by counter-staining with hematoxylin. Sections were observed under Leica DMRB microscope and images were captured with a canon Power shot S 70 digital camera. Positive stained area of the section were quantified using Fiji software (NIH, Bethesda, USA) (Crowe & Yue, 2019).

Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Himedia Laboratories (Mumbai, India) and maintained in tissue culture flasks coated with 0.5%

gelatin in HiEndoXL Endothelial Growth medium supplemented with HiEndoXL Endothelial Growth Supplement and 1X antibiotic antimycotic solution. The cultures were maintained at 37°C and 5 % CO_2. Cells were routinely sub-cultured using EnVzyme Easy at a confluency of 70-80%. Briefly, residual medium from culture flask was discarded and adherent cells were washed with pre-warmed (37°C) PBS. Cells were incubated with prewarmed EnVzyme Easy at 37°C for 7-8 min for cells to detach from surface of the tissue culture flask. The detached cells were then resuspended in prewarmed culture medium and centrifuged at 1000 rpm for 2 min. Cell pellet was resuspended in fresh prewarmed culture medium and seeded in fresh gelatin coated tissue culture flask &/or well-plates for experimentation in a ratio of 1:3. Exponentially growing cells of passages 2-6 were used for all experiments and cells in passage 1-3 were frozen in DMEM containing 30% FBS and 7% DMSO for storage in liquid nitrogen until further use. Revival of the cryopreserved cells resulted in 50-60% efficiency.

Human monocyte (THP-1) cell line was obtained from National Centre for Cell Science (NCCS), Pune, Maharashtra, India and maintained in RPMI-1640 medium supplemented with L-glutamine (2 mmol/l), 10% FBS and 1X antibiotic antimycotic solution in a humidified atmosphere with 5% CO_2 at 37°C. Culture medium of the tissue culture flask was replaced every 2-3 days until cells reached a maximum density of 1.5-1.8 x 10^6 cells/ml. At this density, cells were collected and centrifuged at 1000 rpm for 2 min. Residual medium was removed and cells were resuspended in fresh culture medium. Cells were seeded in tissue culture flask at a density of 2-4 x 10^5 cells/ml and residual cell suspension was used for experimentation. Exponentially growing cells of passages 22-35

were used for all experiments. Cells were cryopreserved in RPMI-1640 containing 30% FBS and 5% DMSO and stored in liquid nitrogen until further use.

THP-1 differentiation

PMA was used for differentiation of THP-1 monocytes and the dose was determined by checking the % adherence and macrophage marker expression (CD68, CD71 for M0 and CD14 for M1) by semi-quantitative RT-PCR (Genin et al., 2015; Park et al., 2007). Briefly, THP-1 cells (1.5×10^6 per ml) were seeded in serum-free RPMI-1640 with varying concentrations (0-100 nM) of PMA for 24 h. After incubation, non-adherent cells were collected, centrifuged and pellet was resuspended in 200 µl PBS. Number of non-adherent cells were counted and % adherence was calculated relative to the number of cells seeded initially. Monocytes differentiated using 50nM and 100nM PMA showed >70% adherence (Fig. M1a) and upregulation of macrophage markers CD71 and CD68. But, exposure to 100nM PMA upregulated CD14 expression indicating M1 polarization that was not observed at 50nM dose (Fig. M1b).

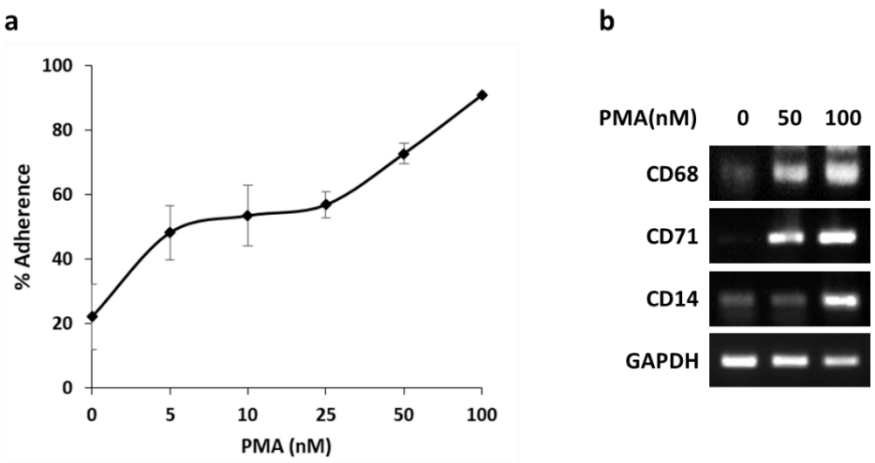

Figure M1: Standardization of PMA induced THP-1 differentiation. (a) THP-1 monocytes were treated with varying concentrations of PMA (0-100nM) for 24 h and % adherence was determined. Data represents mean ±SEM (N=3). (b) mRNA expression of macrophage markers (CD68, CD71, CD14) were analyzed by RT-PCR in THP-1 monocytes differentiated using 50nM and 100nM PMA for 24 h.

For all further experiments, THP-1 monocytes were differentiated with 50nM PMA for 24 and the adherent monocyte derived macrophages (MDMs) were washed with phosphate buffered saline (PBS) before experimental treatments.

Plasmids and shRNA

pCMV3-HSPD1-GFPSpark plasmid (Cat. HG11322-AG) was purchased from Sino Biological Inc. (China). The physical map of the plasmid is shown in Fig. M2. The plasmid contains full length clone DNA of *hspd1* (encodes for human HSP60) with C-terminal GFPSpark tag under the Enhanced CMV promoter. pcDNA3.1-HSP60-MycHis plasmid, obtained as a kind gift by Dr. Thomas Corydon (Aarhus University, Denmark), contains full-length human HSP60 cDNA subcloned in pcDNA3.1-MycHis-A vector at BamHI/ApaI site (Fig. M3).

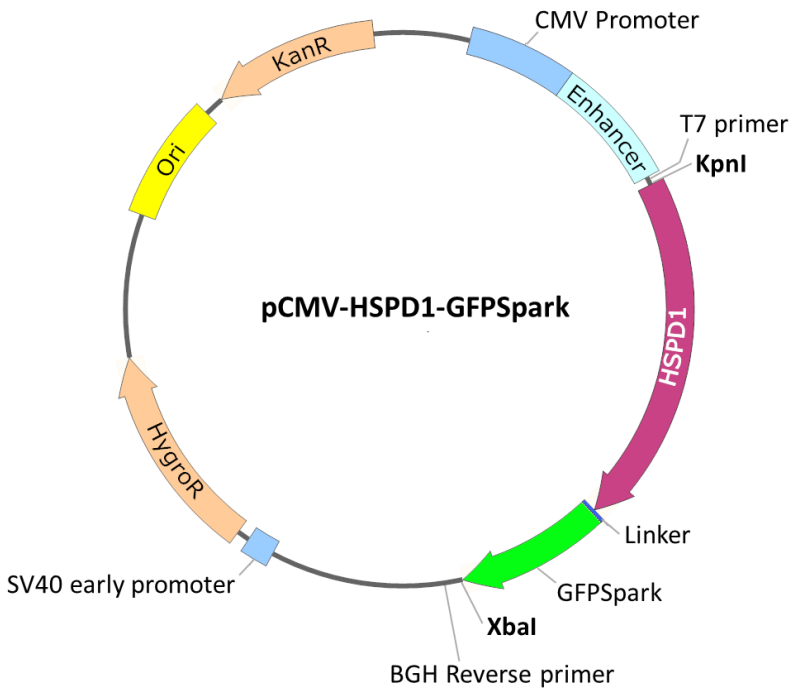

Figure 10: Physical map of pCMV-HSPD1-GFPSpark

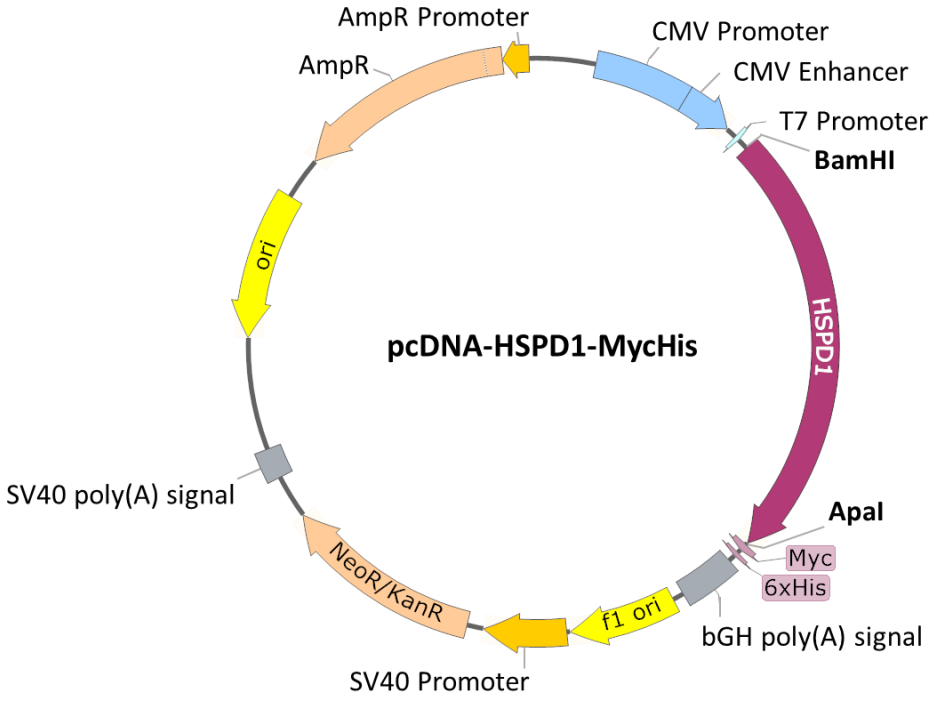

Figure 11: Physical map of pcDNA3.1-HSP60-MycHis

pshRNA-609 containing shRNA sequence against *hspd1* cloned between BamHI and HindIII sites of p*Silencer* 2.0-U6 vector was also a kind gift from Dr. Thomas Corydon (Aarhus University, Denmark). The shRNA is a 65-nucleotides long complementary sequence that contains 19-mer hairpin sequences specific to the HSP60 mRNA, a loop sequence separating the two complementary domains, a poly (T) tail for transcriptional termination. The sequence of shRNA showing 19-mer complementary domains (underlined) and BamHI/HindIII overhangs is as follows:

shRNA-609: 5'-GATCCCGTGCTCACCGTAAGCCTTT
GTTCAAGAGACAAAGGCTTACGGTGAGCATTTTT
TGGAAA-3'

HSP60 overexpression

For HSP60 overexpression in HUVEC, cells were transfected with either pCMV3-HSPD1-GFPSpark (HSP60-GFP) or pcDNA3.1-Hsp60-MycHis (pcDNA-HSP60) plasmids. Cells transfected with either pCMV3-C-GFPSpark (Cat. CV027; Sino Biological Inc., China) or pcDNA3.1-MycHis-A (Cat. V80020; Thermo Fisher Scientific, USA) empty vectors were used as negative control (Vector). Briefly, cells were seeded in respective well-plates and allowed to reach 70-80% confluency. 1 h prior to transfection, the culture medium in well-plates was replaced with prewarmed Opti-MEM medium. The cells were transfected with plasmids using lipofectamine 3000 reagent (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. 4 h after transfection, the medium was removed and cells were washed thoroughly with prewarmed PBS. Fresh prewarmed endothelial growth medium devoid of antibiotic antimycotic solution was added to the wells and cells were incubated at 37°C with 5% SCO_2 in humidified atmosphere for 24 h. The transfection efficiency was determined by observing the cells using green filter of FLoid Cell Imaging station (for

plasmid tagged with GFPSpark) and by assessing HSP60 mRNA and protein expression (for both the plasmids). Cytotoxic effects on the cells was determined by MTT assay.

HSP60 Knockdown

For HSP60 knockdown, HUVEC and THP-1 MDMs were transfected with pshRNA-609. p*Silencer* 2.0-U6 Negative Control (Cat. AM#7209; Thermo Fisher Scientific, USA) containing scrambled shRNA sequence lacking homology to human genome was used as negative control. For HUVEC, cells were seeded in respective well-plates and allowed to grow till 70-80% confluency. 1 h prior to transfection, the culture medium was replaced with prewarmed Opti-MEM. For THP-1, cells were differentiated to MDMs using PMA 24 h and cells were washed with prewarmed PBS. Prewarmed serum free RPMI-1640 was added to the wells 1 h prior to transfection. pshRNA-609 or negative control plasmid was transfected in HUVEC or THP-1 MDMs using lipofectamine 3000 (Thermo Fisher Scientific, USA) according to manufacturer's protocol. After 4 h of transfection, cells were washed 3-4 times with prewarmed PBS and incubated in respective growth medium devoid of antibiotic antimycotic solution for 48 h. The transfection efficiency was assessed by checking HSP60 mRNA and protein expression and cell viability was determined by MTT assay. HSP60 KD cells were further exposed to OxLDL (80 µg/ml) in serum-free medium for 24 h (HSP60 KD + OxLDL).

Cell viability assay

Cell viability at various stages of experimentation was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. For MTT assay, HUVEC cells were seeded in gelatin coated sterile 24 well plate whereas THP-1 cells were directly differentiated using PMA in 24 well plate. After appropriate treatments or transfections,

the cells were washed with PBS, followed by addition of 0.5 mg/ml MTT in serum free medium to each well and incubation at 37°C for 4 h. The purple formazan crystals formed were dissolved in DMSO and the absorbance was recorded at 590 nm using Synergy HTX Multimode Microplate Reader (BioTek Instruments Inc., USA). The results were represented as percentage cell viability with respect to control.

Nitric Oxide Production

Nitric oxide (NO) in plasma or other physiological fluids or buffers is oxidized almost completely to nitrite, where it remains stable for several hours (Kelm, 1999). Thus, levels of nitric oxide (NO) produced can be determined by measuring its oxidized form (nitrite) using Griess' reagent (Green et al., 1982). Briefly, conditioned medium from cells of control and experimental groups was collected and centrifuged at 800xg for 5 min to remove debris. The clear supernatant was mixed with equal volume of Griess' reagent (Sigma-Aldrich, USA) and the mixture was incubated in dark for 30 min at RT. Absorbance was recorded at 540 nm using Synergy HTX multimode reader (BioTek Instruments, USA). Sodium nitrite was used to prepare standard curve and the concentration of nitrite in samples was determined from the standard curve using GraphPad Prism 6.0.

HSP60 secretion

The secretion of HSP60 was checked in conditioned media from the cells using RayBio® Human HSP-60 ELISA kit according to manufacturer's instructions. Briefly, conditioned media from cells was collected and centrifuged at 800xg for 5 min to remove cellular debris. The clarified supernatant was added to wells and incubated for 2.5 h at RT followed by washing four times with wash buffer. Biotin-labelled anti-HSP60 antibody was added

to the wells and incubated for 1 h at RT. Residual anti-body was removed and wells were washed four times using wash buffer, followed by addition of streptavidin-linked secondary anti-body for 45 min. Residual antibody was removed and wells were washed four times using wash buffer. Substrate reagent was added and incubated for 30 min in dark followed by addition of stop reagent. The absorbance was recorded at 450nm using Synergy HTX multimode reader (BioTek Instruments, USA). ELISA of recombinant HSP60 standards was conducted to obtain a standard curve and concentration of HSP60 in samples were extrapolated from the standard curve using GraphPad Prism 6.0.

Immunofluorescence staining

Intracellular and surface immunofluorescence staining was carried out as per protocol described by Jakic et al. (2017) with minor modifications. HUVEC were grown on gelatin coated sterile coverslips and subjected to HSP60 overexpression (pcDNA-HSP60) or OxLDL treatment for 24 h. For intracellular staining, cells were washed with warm PBS and fixed with 2% PFA for 10 min at RT followed by 100% methanol (MeOH) for 10 min at RT for intracellular staining. Cells were washed thrice with wash buffer (0.5% BSA in PBS) followed by blocking with 1% BSA in PBS for 1 h at RT. Cells were incubated overnight with HSP60 rabbit monoclonal antibody (1:200; Cell Signaling Technology, USA) at 4˚C in humidified chamber. After washing thrice with wash buffer, cells were incubated with Alexa Fluor-488 anti-rabbit secondary antibody (Invitrogen, Thermo Fisher Scientific, USA) for 1 h in dark at RT. Cells were washed thrice in wash buffer and coverslip was mounted using Fluoroshield with DAPI (Sigma-Aldrich, USA).

For surface staining, cells were fixed with 1% PFA for 10 min at RT, washed with washed buffer and blocked with 1% BSA in PBS for 1 h at RT. Cells were incubated overnight

with HSP60 rabbit monoclonal antibody at 4˚C in humidified chamber, washed and incubated with Alexa Fluor-488 anti-rabbit secondary antibody for 1 h at RT in dark. Cells were washed thrice with wash buffer and subsequently incubated with β-actin mouse monoclonal antibody (1:100; Invitrogen, Thermo Fisher Scientific, USA) at 4˚C in humidified chamber in dark. After washing thrice, Alexa Fluor-568 anti-mouse secondary antibody (Invitrogen, Thermo Fisher Scientific, USA) was added for 1 h RT in dark. After washing, coverslip was mounted in Fluoroshield with DAPI. For negative controls, the primary antibodies were excluded. Immunofluorescent cells were observed using Zeiss LSM 710 confocal microscope (Carl Zeiss AG, Germany) and images were captured. Images were processed and analyzed using ImageJ (NIH, Bethesda, USA). The negative controls did not show any fluorescence.

For mitochondrial co-localization, HUVEC were stained with 200nM MitoTracker Red CMXRos in serum free medium at 37°C for 40 min in dark. All the subsequent steps were carried out in dark. The cells were washed twice with warm PBS and fixed with 2% PFA for 10 min at RT followed by 100% methanol (MeOH) for 10 min at RT. Further, the cells were subjected to intracellular immunostaining using HSP60 rabbit monoclonal antibody and Alexa Fluor-488 anti-rabbit secondary antibody as mentioned above, with final mounting in Fluoroshield with DAPI. Cells were observed for immunofluorescence and images were captured using Zeiss LSM 710 confocal microscope (Carl Zeiss AG, Germany). Images were analyzed using ImageJ (NIH, Bethesda, USA). Negative control where primary antibody was excluded, did not record any fluorescence of Alexa fluor - 488.

Quantification of immunofluorescence

For quantification of HSP60 immunofluorescence, the fluorescent images (single channel) were analyzed using ImageJ as described by Jakic et al. (2017). In every image, each cell was selected and its area, integrated density and mean gray value were measured. Also, mean gray values (fluorescence intensities) of four different background areas were measured in every image for normalization of background autofluorescence. The corrected total cell fluorescence (CTCF) for each cell was calculated as:

CTCF= Integrated density – (area of cell x mean of background fluorescence)

The intensities were calculated for more than 80 cells from a total of 5-10 different images for each experimental sample and the values were used for statistical analysis.

Monocyte endothelial adhesion assay

Monocyte endothelial cell adhesion assay was carried out as described by Yanaka et al. (2011) with minor modifications. HUVEC were seeded in gelatin coated 12 well plate and allowed to reach ~80% confluence, after which the cells were transfected for HSP60 overexpression or knockdown as described earlier or treated with OxLDL (80µg/ml) for 24 h. THP-1 monocytes were labeled with 1 µg/ml Hoechst 33342 in incomplete medium for 15 min in dark. After washing with warm PBS twice, the Hoechst labelled THP-1 monocytes (0.5×10^5 cells/well) were added to the wells containing HUVEC and incubated for 30 min. The unbound monocytes were removed by aspiration of residual medium followed by washing the wells with warm PBS thrice. Monocytes bound on HUVEC were observed using FLoid Cell imaging station (Thermo Fisher Scientific, USA) and images

were captured. Number of HUVEC and bound THP-1 monocytes were counted and % monocyte adhesion was calculated relative to endothelial cells.

OxLDL uptake study

Lipids accumulation in the cells was monitored by staining intracellular lipid granules with oil red O (ORO) stain. For qualitative analysis, THP-1 cells were seeded on sterile coverslips in 12 well plate and differentiated using 50 nM PMA for 24 h. HSP60 knockdown was achieved in MDMs using pshRNA-HSP60 as mentioned above and MDMs (control and HSP60 KD) were treated with OxLDL (8 and 80 µg/ml) for 24 h. The cells were fixed in 4% PFA for 45 min, washed with PBS and stained with 0.5% ORO for 30 min at RT. After washing with distilled water, coverslips were mounted on slides and observed under Leica DM750 microscope (Leica Microsystems, Germany) and images were captured. For quantitative analysis, the same protocol was run in 12 well plate without coverslip. The ORO stain accumulated in cells was extracted in 50% isopropanol and absorbance was recorded at 495nm using Synergy HTX Multimode Microplate Reader (BioTek Instruments Inc., USA). % ORO accumulation was calculated relative to untreated KD-Control and graph was plotted using GraphPad Prism 6.0.

Mitochondrial membrane potential

The mitochondrial integrity was monitored by detecting the change in mitochondrial membrane potential ($\Delta\Psi m$) using lipophilic fluorescent probe JC-1. JC-1 is a membrane permeable dye that exhibits potential-dependent accumulation in mitochondria leading to formation of aggregates. The formation of J-aggregates in a healthy mitochondria results in shift of its fluorescence maxima from green (~529 nm) to red (~590 nm) making it an indicator of mitochondrial health (Smiley et al., 1991). Briefly, THP-1 cells were

differentiated to MDMs in 12 well plate using PMA as per the protocol mentioned above. MDMs were transfected with p*Silencer* 2.0-U6 Negative Control (KD-Control) or pshRNA-609 (HSP60 KD) for 48 followed by treatment with OxLDL (8 and 80 µg/ml) for 24 h. Thereafter, the cells were incubated with 5 µM JC-1 dye in incomplete RPMI-1640 medium for 20 min at 37˚C. Cells were washed with warm PBS (37°C) to remove excess dye and fluorescent images of JC-1 monomer (green fluorescence) and JC-1 aggregates (red fluorescence) were captured using FLoid Cell Imaging station. Total integrated intensity of green- and red-fluorescence in the individual cells was measured using Image J (NIH, Bethesda, USA) as mentioned above and red/green fluorescence ratio was calculated. The ratio of red fluorescence to green fluorescence was considered as a change in the Mitochondrial Membrane Potential ($\Delta\Psi m$).

RNA isolation and cDNA synthesis

Total RNA was isolated from thoracic aorta, HUVEC and THP-1 samples using Trizol reagent (Invitrogen, Thermo Scientific, USA) following manufacturer's protocol. Tissue was homogenized in Trizol reagent whereas, the cells were directly lysed using Trizol reagent in the culture dish. Lysis/homogenization was followed by phase separation using chloroform. RNA was precipitated from aqueous phase using pre-chilled isopropanol, centrifuged to pellet that was subsequently washed once with pre-chilled 75% ethanol. The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water and the purity of same was assessed by measuring A260/A280 ratio in UV-Vis spectrometer (PerkinElmer, USA). Samples with A260/A280 within the range of 1.8-2.0 was used for gene expression studies. Samples were quantified using absorbance at 260 nm as follows:

Concentration (µg/ml) = A260 x 40 x dilution factor

where, 40 = extinction coefficient (40 µg/ml of RNA= 1 absorbance)

1 µg of RNA was used to synthesize cDNA by reverse transcription using iScript cDNA synthesis kit (Bio-Rad Laboratories, USA) according to manufacturer's protocol and cDNA was used as template for quantitative RT-PCR.

Quantitative RT-PCR

Quantitative RT-PCR was performed using Power Up SYBR Green Master Mix (Thermo Fisher Scientific, USA) as per the manufacturer's protocol and the reaction was run on the QuantStudio-3 Real-Time PCR System (Applied Biosystem, Thermo Fisher Scientific, USA). mRNA expression of the genes of interest were analyzed using $2^{-\Delta\Delta CT}$ method using GAPDH as endogenous control. Primers used for this study are listed in Table 4.

Table 4: Primer sequences for quantitative PCR

Gene Name	***Forward Primer (5'→3')***	***Reverse Primer (5'→3')***
Human		
eNOS	CCTCGTCCCTGTGGAAAGAC	GTGGTCCACGATGGTGACTT
MCP-1	TCTGTGCCTGCTGCTCATAG	CTTCTTTGGGACACTTGCTGC
VCAM-1	GGGAAGCCGATCACAGTCAA	GGGACTTCCTGTCTGCATCC
ICAM-1	GCTGTCTACTGACCCCAACC	GGTGACCTTGAATGTGACATGG
SRB1	GAAGGCATCCCCACCTATCG	AATTCCAGACTCCAGGCACG
SRA1	AAAGTTCGACTGGTCGGTGG	CCCAAGCTCCTACAGACGAC
CD36	CGAGGAAGCCACTTTGGTGA	TGGTTTCTACAAGCTCTGGTTCT
iNOS	CGCATGACCTTGGTGTTTGG	CATAGACCTTGGGCTTGCCA
IL-6	ACCCCCAGGAGAAGATTCCA	GATGCCGTCGAGGATGTACC
ARG-1	GGGTTGACTGACTGGAGAGC	CGTGGCTGTCCCTTTGAGAA
IL-10	AGCTCCAAGAGAAAGGCATCT	TCGCCACCCTGATGTCTCA
HSP60	GTTGGGGGACCGCTCATT	CCCGGCCATCCTTATAGACG
HSP10	AGTAGTCGCTGTTGGATCGG	TTGGTGCCTCCATATTCTGGG
HSF-1	GAACAGCAGCCCGGATTCAG	AGAAGTAGGAGCCCTCTCCC
GAPDH	GAGTCAACGGATTTGGTC	GACAAGCTTCCCGTTCTC
Mouse		
Col I	GAGAGGTGAACAAGGTCCCG	AAACCTCTCTCGCCTCTTGC
Col III	AACCAAGGCTGCAAGATGGA	TGTCCACCAGTGCTTACGTG
eNOS	TGGAAGGGAAGTGCAGCAAA	GGCCAGTCTCAGAGCCATAC
MCP-1	TGACCCCAAGAAGGAATGGG	GACCTTAGGGCAGATGCAGTT
VCAM-1	CTGGGAAGCTGGAACGAAGT	GCCAAACACTTGACCGTGAC
ICAM-1	GTGGGTCGAAGGTGGTTCTT	AAACAGGAACTTTCCCGCCA
HSP60	TGATGTTGGCTGTGGATGCT	GACACCCTTTCTTCCAACCTTT
HSP10	TTGGTTGAAAGGAGTGCTGC	TGACAGGCTCAATCTCTCCAC
HSF-1	AGCTCCATCTCCAGCCTACA	CATCTATGCTCCTGCCTGGG
GAPDH	GTCGGTGTGAACGGATTTGG	TAGATGCCTGCTTCCCATTC

Western blotting

For extracting total protein lysate, tissue was homogenized in RIPA buffer (50 mM tris (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% triton-X-100) containing PIC and 1 mM PMSF followed by incubation in ice-bath for 2 h. For HUVEC and THP-1 samples, cell pellets were washed twice with ice-cold PBS, lysed in RIPA buffer and incubated for 30 min in ice-bath. The tissue and cell lysates were centrifuged at 10,000 rpm at 4˚C for 20 min and resultant supernatant was subjected to protein estimation using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, USA). Equal quantity of protein from each sample was denatured in 6X loading dye at 95°C-100°C for 5 min. 25 µg of each protein sample was separated by SDS-PAGE and subsequently transferred onto PVDF membrane using standard protocol of Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA). Transfer of proteins was checked by staining membrane with 0.05% Poncaeu S. Membrane was destained in distilled water followed by blocking with 3% BSA in Tris buffered saline (TBS) for 1 h at RT. The membrane was incubated overnight in anti-HSP60 (1:1000), anti-HSP10 (1:1000) and anti-β-actin (1:1000) antibodies in 3% BSA. After washing thrice with TBS containing 0.1% Tween 20 (TBST), the membrane was probed with HRP-linked anti-rabbit secondary antibody (1:2000) for 1 h at RT. Expression of the immune-reactive proteins was detected using Clarity^{TM} Western ECL Substrate (Bio-Rad Laboratories, USA) according to the instruction manual and chemiluminiscence was captured using iBright CL1000 Imaging Station (Invitrogen, Thermo Fisher Scientific, USA).

Statistical Analysis

All the statistical analysis were carried out using GraphPad Prism 6.0. Differences between two groups were compared by unpaired two-tailed Student's t-test and for more than two group with one variable, one-way ANOVA followed by Tukey's test was carried out. For analysis of data containing two independent variables, differences were compared using Two-way ANOVA followed by Tukey's test. Results were expressed as mean ± S.E.M. For all analysis, differences were considered statistically significant at *P <0.05, **P<0.01 and ***P <0.001.