Online Supplement:

Supplement Methods:

Isolation of Human Peripheral Blood Monocytes (PBMC*).* 50–60 ml of blood from adult normal healthy donors were collected in the presence of anticoagulant in accordance with an approved Institutional Review Board protocol. Monocytes were isolated as described earlier (1). Briefly, an equal volume of diluted blood was overlaid on Ficoll-Paque-plus in 1:1 ratio and centrifuged at 400x *g* for 20–30 min at 18–20 °C. The leukocyte population was collected from the interface and washed. About 50 million washed cells in 10 ml of RPMI medium containing 10% FCS were plated in 100-mm culture dishes to allow monocytes to adhere on the surface of the dish for 2–3 h. The adherent cells which represent the monocyte population were washed twice with warm RPMI medium containing 10% FCS and allowed to remain in the dish overnight at 37 °C in 5% CO2. During this period the monocytes detach from the dish. They were collected and washed in fresh RPMI medium and labeled as PBMC. Then, about $1X10^5$ cells from normal volunteers per well in 6-well plates were treated with MDA-Lys for 4 h, and total RNA isolated as described below.

Synthesis and purification of MDA-Lysine adduct: Malondialdehyde (MDA) was prepared by hydrolysis of malonaldehyde bis-(dimethyl acetal) with HCl for 35 min at 40ºC. The resulting dark yellow solution was cooled to 10ºC and then pH was adjusted to 7.0 with saturated bicarbonate solution. Sodium cyanoborohydride was added followed by Boc-Lysine in acetonitrile-water (1:1) mixture and stirred at 25ºC for 24 hours. Sodium borohydride was then added and stirred overnight. The reaction mixture pH was then adjusted to pH 2 with 1M HCl and lyophilized. The lyophilyzed sample was redissolved in 30 ml of 2M HCl and again lyophilized. The lyophilized sample was resuspended in water and applied to a Dowex 50H+ cation exchange

chromatography column, washed with water and eluted with 0.5M ammonia. Eluted crude product was analyzed by mass spectrometry and fractions containing MDA-Lys were pooled and loaded on UNOsphere Cation Exchange column, washed with water and MDA-Lys eluted with a gradient of formic acid-water (0.05M – 1M). Fractions were analyzed by MS. Fractions containing the pure product were pooled, lyophilyzed, dissolved in water, aliquoted and stored at -20^{o}C.

Determination of MDA-Lys induced ROS and Superoxide Formation: MDA-Lys-induced ROS and superoxide production were detected using H_2DCFDA or dihydroethidine staining respectively as described earlier (2). Briefly, THP-1 cells were seeded in 12-well dishes at 50,000 cells per well. Cells were serum-starved for 12h and then treated with MDA-Lys for 30 min and stained for superoxide formation with dihydroethidine (20μmol/L), for ROS formation with H2DCF-DA (20μmol/L) for 10 min at 37 °C. The cells were then washed with phosphate-buffered saline. Fluorescence was detected using a fluorescence microscope (wavelength: 485/530 nm or 518/605nm) and quantified using Image Pro software (Media Cybernetics, Inc., Silver Spring, MD).

Preparation of Cell Lysates and Western Blotting: After stimulation with MDA-Lys, total cell lysates were prepared in SDS sample buffer for Western blotting. Protein samples were fractionated on 12.5% SDS-polyacrylamide gels, then transferred to nitrocellulose membranes, immunoblotted with the indicated antibodies, and blots developed using chemiluminescence reagents as described earlier (3).

Preparation of Nuclear protein extracts and Electro Mobility Shift Assays (EMSAs): Nuclear extracts from each of NG and MDA-Lys treated samples were prepared as described (1). Binding reactions were carried out in 20 μl reaction volume with ^{32}P-labeled NF-kB, AP-1 or Egr-1 consensus oligonucelotides (10pmol) and incubated with

corresponding extracts for 15 min at room temperature in a binding buffer containing 10mM Tris-Cl (pH 7.5), 100mM potassium acetate, 2mM magnesium acetate, 1mM DTT, 10mM creatine phosphate, 1mM ATP and 0.1mM spermine. To the reaction mix 30 μg/ml of heparin was added and incubated for a further 10 min to reduce non-specific binding. The samples were subsequently resolved by 10% native PAGE and visualized by autoradiography.

RNA Preparation and Relative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): Total RNAs were prepared from NG or MDA-Lys treated THP-1 cells by RNAeasy method. Total RNA was isolated from THP-1 cells ($2x10^6$/sample) grown in NG or MDA-Lys for 1- 24h and 1 μg RNA used for the RT reaction using Gene Amp RNA PCR kit. cDNA corresponding to 0.05 μg of RNA was then used in multiplex PCR reactions containing gene-specific primers (Supplement Table-1) paired with Quantum18S RNA Internal Standards. Multiplex PCR reactions were performed for 30-35 cycles in a GeneAmp9700 machine (Applied Biosystems, Inc., and Foster City, CA City). PCR products were fractionated on 2.5% agarose gels, photographed using AlphaImager 2000 and quantified with Quantity One software (Bio-Rad Laboratories, Hercules, CA). Results were expressed as fold stimulation over NG after normalizing with paired 18S RNA levels.

Real time Quantitative PCR (qPCR) was performed on the Applied Biosystems 7300 real-time PCR system (Foster City, CA) using mRNA samples. Human CCL11, CCL18 and TNFSF14 primers for real-time PCR were purchased from Qiagen. Primers for control GAPDH shown in Supplement Table-1. Each sample was run in triplicate. The relative RNA amount was calculated and normalized with the internal control,

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), according to the method described earlier (1).

Cytokine Antibody Array Analysis: The expression profile of MDA-Lys-induced cytokines in THP-1 cells was examined with Human Cytokine antibody Arrays (Ray Biotech). 1ml of conditioned serum free media from $1X10^7$ THP-1 cells cultured in a six well plate either with or without MDA-Lys (10μg/ml) for 12h were hybridized to three identical cytokine antibody array membranes according to manufacturer's instructions. Each antibody array consists of antibodies to 120 known cytokines spotted in duplicate onto a charged nylon membrane. The blotted membranes were scanned and specifically bound illuminant spots were quantified using Image Quant software (Molecular Dynamics, Sunnyvale, CA). Then, the relative percentage changes in cytokine levels were calculated. At least three images from each set of arrays were collected and analyzed. Relative changes in cytokine levels in three independent experiments were calculated and results expressed as fold induction over NG grown cells.

Biological network and pathway analysis using Ingenuity Software For network and pathway analyses, a tab-delimited text file of a data set containing only the significantly up- or down regulated molecules with their corresponding fold changes (Supplement Table-2 and Figs.2 and 3) was uploaded into the Ingenuity software (web-delivered) (IPA). A stringent criterion of cytokine induction of 1.5 fold or above was applied to select differentially expressed genes/proteins. The IPA application mines significant biological networks or pathways from Ingenuity Pathways Knowledge Database (IPKDB) containing large amounts of gene objects (e.g., genes, mRNAs and proteins) with individually modeled relationships between them. The submitted protein or genes that

are mapped to the corresponding gene objects in the IPKDB are called "focus protein or genes." The focus genes are used for generating biological networks. We removed edges with proteolysis, phosphorylation/dephosphorylation and other secondary biochemical modifications. Each network and canonical pathway's *P* value is calculated according to the fit of the set of significant focus proteins or genes. This is done by comparing the number of focus proteins that participate in a given network or pathway relative to the total number of occurrences of those proteins in all networks or pathways stored in the IPKDB.

DNA Transfection and Luciferase Assays: THP-1 cells plated in 6-well plate (1.2×10^6 per well) were transfected with 0.5 μg of the plasmids, p3xNF-kB-Luc (Promega), pIP-10-Luc (-438), pMCP-1-Luc(-3011), and pCOX-2-Luc(-860) using a Nucleofector and kits from Amaxa Inc according to manufacturer's protocols. The transfected cells were cultured for 8h in either NG or MDA-Lys containing medium, and luciferase activity assays performed as described earlier (1; 3).

Monocyte Adhesion Assays: THP-1 cells were cultured in NG or MDA-Lys (10μg/ml) for 12 h. Adhesion experiments were carried out as described earlier (3).

Animal Experiments . All animal care, procedures and protocols were followed according to the policies outlined in "The Guide for the Care and Handling of Laboratory Animals" (NIH Publication No. 85-23), which have been approved by the City of Hope National Medical Center Research Animal Care Committee. Male Sprague-Dawley rats (~175 to 200g) were obtained from Charles River Laboratories (Wilmington, MA, USA). After a one-week adaptation period, rats were rendered diabetic by intraperitoneal injection of STZ (65 mg/kg in citrate buffer, pH 4.5) after an overnight fast. Non-diabetic

animals were injected with the buffer only. Diabetes was confirmed by measuring the plasma glucose concentrations 7 days after STZ-injection. Only animals with plasma glucose concentration of more than 20 mmol were classified as diabetic and were used in the study. Rats were divided randomly into non-diabetic control group (n=10), and diabetic group (n=10). All animals were housed individually and were given free access to food and water. At 32 weeks, blood samples were collected from each animal into heparinized vacutainer tube, and centrifuged for plasma isolation and frozen at -80°C (4).

REFERENCES:

1. Shanmugam N, Ransohoff RM, Natarajan R: Interferon-gamma-inducible protein (IP)-10 mRNA stabilized by RNA-binding proteins in monocytes treated with S100b. *J Biol Chem* 281:31212-31221, 2006
2. Reddy MA, Li SL, Sahar S, Kim YS, Xu ZG, Lanting L, Natarajan R: Key role of Src kinase in S100B-induced activation of the receptor for advanced glycation end products in vascular smooth muscle cells. *J Biol Chem* 281:13685-13693, 2006
3. Shanmugam N, Kim YS, Lanting L, Natarajan R: Regulation of cyclooxygenase-2 expression in monocytes by ligation of the receptor for advanced glycation end products. *J Biol Chem* 278:34834-34844, 2003
4. Figarola JL, Scott S, Loera S, Xi B, Synold T, Weiss L, Rahbar S: Prevention of early renal disease, dyslipidaemia and lipid peroxidation in STZ-diabetic rats by LR-9 and LR-74, novel AGE inhibitors. *Diabetes Metab Res Rev* 21:533-544, 2005

SUPPLEMENT FIGURE-1

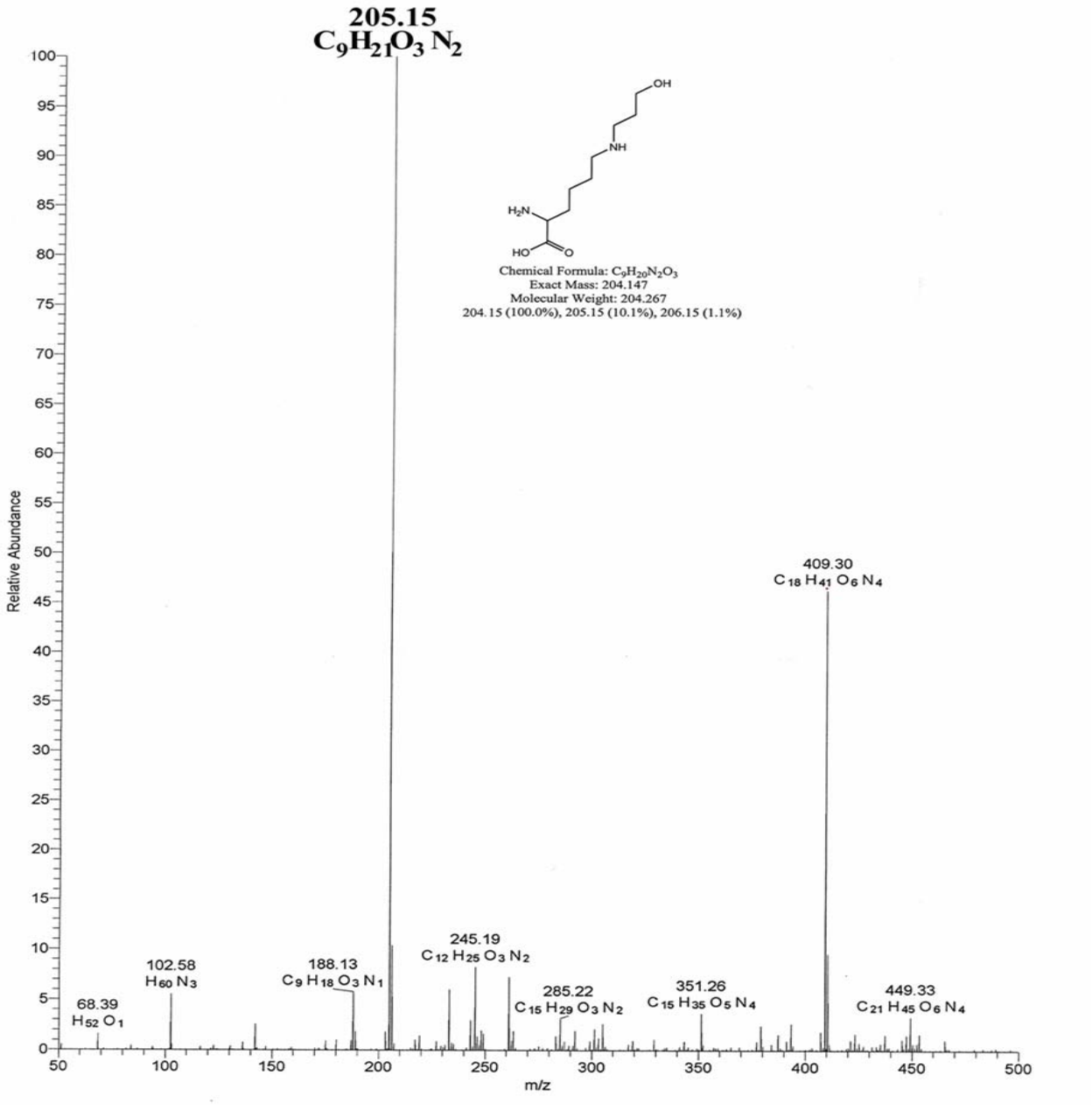

Figure legend:
Electrospray-ionization mass spectrometral profiles of singly and doubly adducted MDA-Lysine adducts. Elution times are shown.

SUPPLEMENT TABLE-1

PCR Primers Sequences and PCR Conditions

	Sequences (5'-3')	Annealing temperature ^{o}C	No. of cycles	Product Size
RAGE				
Sense	AGGAAAGAAGGACACAGAGAC	56	33	264bp
Antisense	ATGGATTTAAGAACCGGCAG			
β_1-Integrin				
Sense	GCCTTACATTAGCACAACACC	60	35	283bp
Antisense	ATCTCCAGCAAAGTGAAACC			
β_2-Integrin				
Sense	AAAAACATCCAGCCCATCTTC	60	35	271bp
Antisense	ATCTGCACGCCATCACAGTC			
β_6–Integrin				
Sense	ACTGCTTTGCCTGTTCTTTC	60	35	167bp
Antisense	TCACACCTTTCGCCAACTC			
CCR2				
Sense	GCCTCATTACCTTGTGCTAATC	60	35	144bp
Antisense	ATCCCTCACTCCCATTCATC			
CD-36				
Sense	GTGACTCATCAGTTCCTTTCC	57	32	222bp
Antisense	GAATACCTCCAAACACAGCC			
COX-2				
Sense	ATCTACCCTCCTCAAGTCCC	64	33	708bp
Antisense	TACCAGAAGGGCAGGATACAG			
COX-1				
Sense	CCGGATGCCAGTCAGGATGATG	64	33	529bp

Antisense	CTAGACAGCCAGATGCTGACAG			
GAPDH				
Sense	GGTGAAGGTCGGAGTCAACG	64	29	251bp
Antisense	CACCATTCTCGCTCCTGGAAGATGGTG			
IP-10				
Sense	CACCAAATCAGCTGCTACTA	59	33	196bp
Antisense	CTGAGAATTCTGATAAACCCCA			
IL-1 β				
Sense	CTCTCTCACCTCTCCTACTCAC	60	33	187bp
Antisense	ACACTGCTACTTCTTGCCCC			
IL-6				
Sense	TCCTGCAGAAAAAGGCAAAG	58	32	249bp
Antisense	GCCCAGTGGACAGGTTTCT			
IL-8				
Sense	AGGGTTGCCAGATGCAATAC	57	32	259bp
Antisense	GCAAACCCATTCAATTCCTG			
iNOS				
Sense	TTTGTTACCGCTTCCACCC	60	35	365bp
Antisense	CAGCAAGCAGCAGAATGAG			
MCP-1				
Sense	CAAACTGAAGCTCGCACTC	60	29	659bp
Antisense	CATTTCCACAATAATATTTTAG			

SUPPLEMENT TABLE-2

MDA-Lys induced changes in cytokines in THP-1 Cells

	Protein	Description	Fold change	Family
1	BTC	Betacellulin	↑ (2.43)	Growth factor
2	CCL1	I-309	↓	Cytokine
3	CCL11	Eotaxin	↑ (4.90)	Cytokine
4	CCL16	HCC-4	↑ (2.74)	Cytokine
5	CCL18	PARC	↑ (4.34)	Cytokine
6	CCL28	CCL-28	↑ (2.78)	Cytokine
7	GRO	Growth Related Oncogene α β & γ	↑ (1.50)	Cytokine
8	CXCL1	GROα	↑ (2.05)	Cytokine
9	CXCL13	BLC	↓	Cytokine
10	HGF	Hepatocyte growth factor	↑ (3.05)	Growth factor

11	IL-15	Interleukin 15	↑ (2.74)	Cytokine
12	IL-16	Interleukin 16	↑ (1.96)	Cytokine
13	IL-1RN	Interleukin 1 receptor antagonist	↑ (1.74)	Cytokine
14	ANG	RNAse A family, 5	↓	Enzyme
15	CSF2	Macrophage colony stimulating	↓	Cytokine
16	IGFBP-4	IGF binding protein 4	↑ (2.02)	Other
17	LEP	Leptin	↓	Growth factor
18	TNFSF14	TNF ligand superfamily member 14	↑ (3.64)	Cytokine
19	CCL2	MCP-1	↑ (2.46)	Cytokine
20	CCL8	MCP-2	↑ (1.51)	Cytokine
21	CCL7	MCP-3	↓	Cytokine
22	CXCL7	NAP-2	↑ (3.32)	Cytokine
23	NTF-3	Neurotrophin 3	↑ (3.78)	Growth factor

↑ = Increase in expression**;** ↓ = Decrease in expression