SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals: Twenty eight-week-old male C57BL/6J mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and housed in colony cages, maintained on a 12-h light/12-h dark cycle. After one week on a maintenance diet (MD, AIN-93G, F3156, BioServ), mice were randomized into two groups and fed either a MD or a high-fat diet (HFD; 60 kcal% saturated fat; F3282, BioServ) for 10 weeks. Body weights and fasted blood glucose levels were monitored every other week and at the end of the study. Resident peritoneal cells were harvested by lavage and plated [1]. After 3 h, non-adherent cells were removed and macrophages were cultured overnight. All studies were performed with the approval of the UTHSCSA Institutional Animal Care and Use Committee.

LDL Isolation: LDL was isolated by KBr-gradient ultracentrifugation from pooled plasma from healthy blood donors and purified by gel-filtration chromatography, filter-sterilized and characterized as described previously [2, 3].

Blood Analysis: Mice were fasted overnight prior to glucose and lipid measurements. Glucose was measured biweekly using a Contour®meter (Bayer). Blood was drawn by cardiac puncture. Plasma cholesterol and triglyceride levels were determined using enzymatic assay kits (Wako Chemicals).

Adenoviral Vectors: To control human glutaredoxin 1 (Grx1) and Nox4 levels and to prevent localization artifacts due to overexpression, we used the previously described doxycycline-controlled Tet-On adenoviral gene expression vector carrying a loxp cassette in its multi-cloning site [1]. Sequences for Grx1-EGFP and Nox4 were first cloned into pDNR donor vectors (Clontech, Mountain View, CA) and then inserted into the adenoviral vector by linear recombination using Cre recombinase (Invitrogen, Carlsbad, CA). THP-1 monocytes were incubated for 24 h with the adenoviruses in RPMI medium supplemented with 10% FBS. Transgene expression was induced by adding doxycycline (1 µg/ml). Infection conditions were optimized to maximize Grx1 and Nox4 expression and to minimize the cytopathic effect of the

adenoviruses. Under these conditions, over 95% of THP-1 monocytes were found to be infected based on the expression of EGFP.

Cell Culture: To mimic metabolic disorders *in vitro*, THP-1 monocytes (2 x 10^6 cells/ml) were cultured at 37°C for 20 h in RPMI 1640 medium containing 10% FBS, 5 mM D-glucose and supplemented with either vehicle, freshly isolated native human LDL (100 µg/ml), D-glucose (HG, 20 mM), or LDL plus HG. Intracellular oxidative stress and thiol oxidation in the absence of LDL or HG was induced by incubating THP-1 monocytes for 2 - 5 h with H_2O_2 (0.1 – 1 mM) in RPMI 1640 medium with 2% FBS. Nox4 knockdown studies were conducted with scramble non-targeting and Nox4 siRNAs purchased from Dharmacon. THP-1 monocytes were transfected for 24 h with siRNAs using GeneSilencer (Genlantis) prior to initiating experiments.

Western Blot Analysis: THP-1 monocyte protein lysates were subjected to Western blot analysis according to standard protocols. Nox4 was identified with highly specific anti-Nox4 monoclonal antibodies [4] (Epitomics). Bands were detected by chemiluminescence on a KODAK Image Station 4000MM and normalized to β-actin (Santa Cruz Biotechnology).

Monocyte Chemotaxis Assay: THP-1 monocytes (2 x 10^6 cells/ml) were pretreated with either vehicle, LDL, HG, LDL+HG, or treated with H_2O_2, and loaded into the upper wells of a 48-well modified Boyden chamber (NeuroProbe). The lower wells contained either vehicle, MCP-1 (R&D Systems), PDGF-B (R&D Systems) or RANTES (R&D Systems). A 5 µm polyvinyl pyrrolidone-free polycarbonate filter membrane was layered between the upper and lower chambers, and the chamber was incubated for 3 h at 37°C and 5% CO_2. The membrane was washed and cells removed from the upper side of the filter. Transmigrated cells were stained with Diff-Quik® Set (Dade Behring, Newark, DE) and counted in five separate high power fields at 400X magnification under a light microscope.

Flow Cytometry: Intracellular H_2O_2 production was measured in cells loaded for 1 h with 20 µM DCFH-DA (Invitrogen), a redox sensitive dye that reacts preferentially with H_2O_2 and other peroxides [5, 6]. Cells were washed and analyzed by FACS (FACSCalibur System, Becton Dickinson). Cell surface expression of CCR2 on THP-1 cells was determined with Alexafluor®647-conjugated mouse anti-human CCR2 antibodies (CD192; BD Pharmingen). After staining for 1 h, cells were washed extensively, fixed with cold 1% paraformaldehyde solution for 5 min and analyzed by FACS.

Monocyte F-actin/G-actin Ratios: THP-1 monocytes (1 x 10^6 cells) were pre-incubated with either vehicle or LDL+HG for 24 h and stimulated for 30 min with either vehicle or MCP-1. Cells were lysed, homogenized and the total cellular actin pool was separated by ultracentrifugation into globular actin (G-actin) and filamentous actin (F-actin) using the G-actin/F-actin *in vivo* Assay Kit (Cytoskeleton). G-actin and F-actin fractions were then separated by SDS-PAGE and actin was quantified by Western blot analysis.

Macrophage Protein-Glutathione Mixed Disulfide Analysis: Resident peritoneal cells were harvested, lysed, and protein-bound glutathione was released by DTT and quantified by HPLC as described elsewhere [7]. Values were normalized to DNA content.

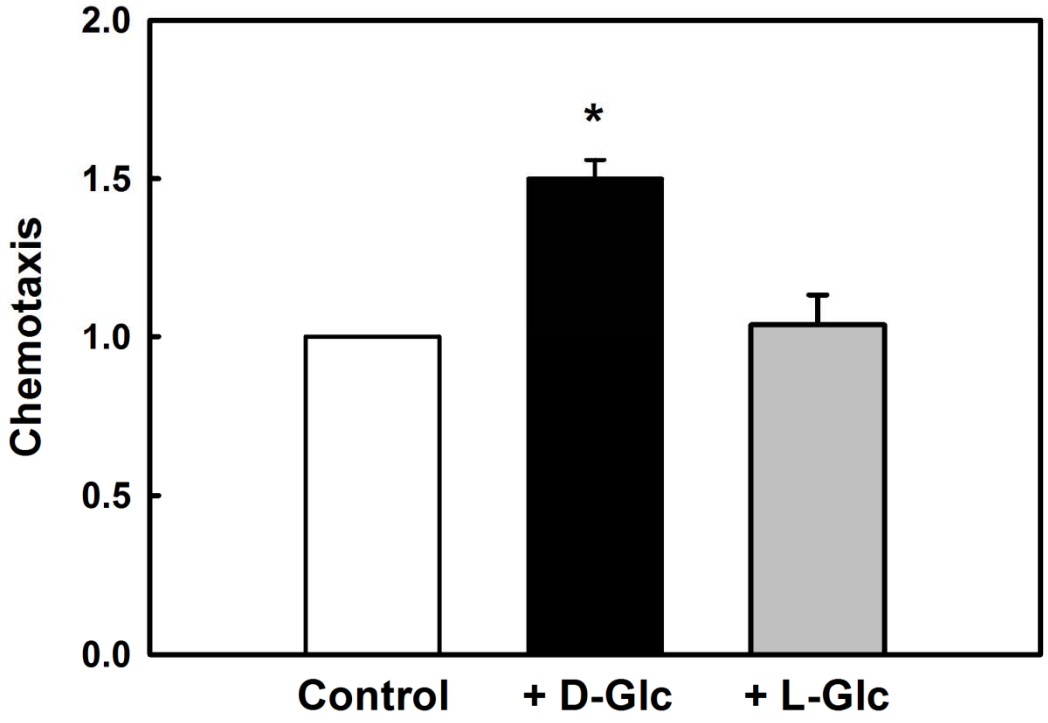
***In Vivo* Matrigel Macrophage Recruitment Assay:** Three days prior to sacrifice, each mouse received two Matrigel plugs as described previously [8]. Briefly, growth factor-reduced Matrigel (BD Biosciences) supplemented with either vehicle or MCP-1 (300 ng/ml; 36 nM) was injected subcutaneously into the right and left flank of each mouse. The plugs were surgically removed at the time of sacrifice, dissolved, and cells were stained with calcein/AM and propidium iodide and counted automatically on a video-based, fluorescence cell counter (Nexcelom Bios, MA). Cell staining with antibodies directed against macrosialin/CD68 confirmed that >93% of the cells recruited into the Matrigel plugs were macrophages.

Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Power SYBR Green Cell-to-Ct Kits (Ambion) were used to quantify Nox4 mRNA. Briefly, mRNA of cells recovered from Matrigel plugs was extracted and cDNA was synthesized by reverse transcription. cDNA was subjected to real-time PCR amplification in the cycling condition as described in manufacturer's manual. Dissociation curve analysis was performed at the end of 40 cycles to verify the identity of the PCR product. No signals were detected in no-template controls. The mRNA copy numbers were calculated based on the Ct number of each reaction and normalized to the internal control, GAPDH. The primers used in the assay were as follows: mouse Nox4 forward 5'-TTGCCTGGAAGAACCCAAGT-3'; mouse Nox4 reverse 5'-TCCGACCAATAAAGGCACAA-3'; GAPDH forward 5'-TGACGTGCCGCCTGGAGAAA-3'; GAPDH reverse 5'-AGTGTAGCCCAAGATGCCCTTCAG-3'.

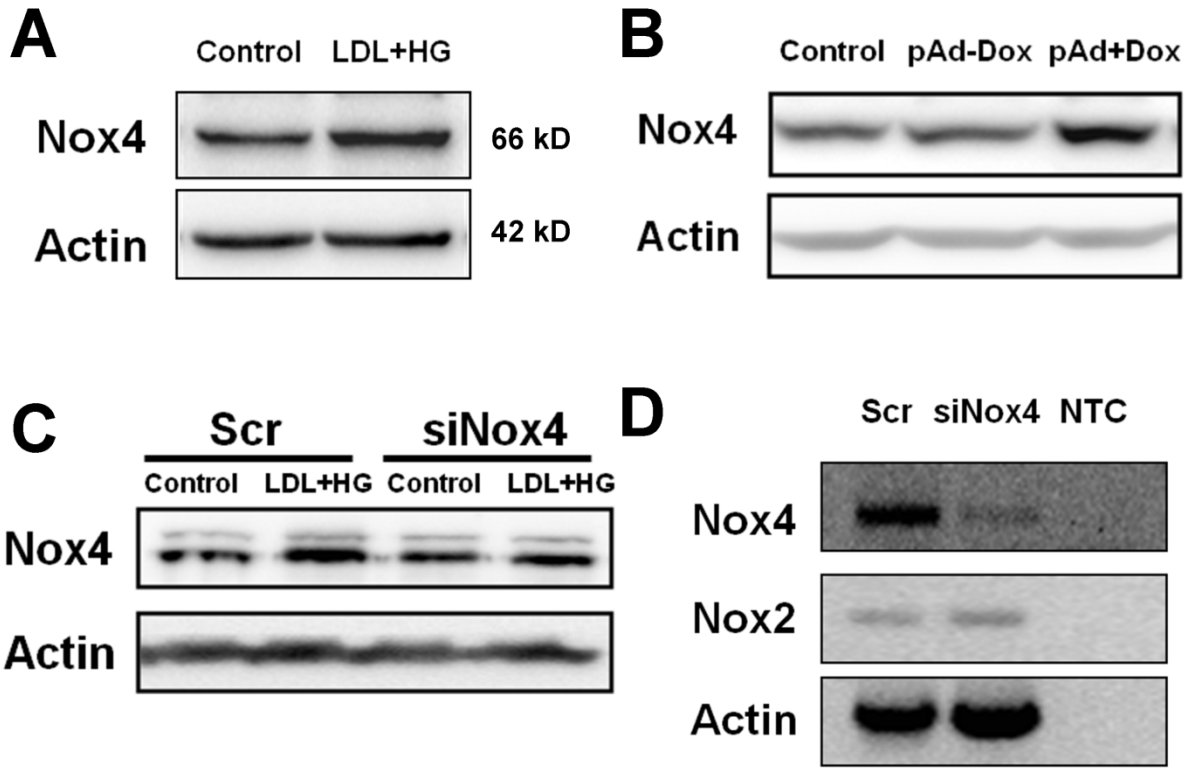
Confocal Microscopy: Human monocyte-derived macrophages (HMDM) were prepare as described in previously [4, 9]. HMDM were fixed in 4 % paraformaldehyde and permeabilized with 0.01 % of Triton X-100. 1 % BSA and 4 % donkey serum were used for blocking. Cells were stained with rabbit anti-Nox4 monoclonal antibodies [4] (1:1000 dilution), Alexa 488-labeled

phalloidin (Invitrogen, 1:500 dilution) and DAPI. Secondary anti-rabbit IgG Cy5 antibodies were from JacksonImmuno Research. Confocal images were collected with an Olympus FV-1000 Laser Scanning Confocal Microscopy at the Optical Imaging Facility of UTHSCSA.

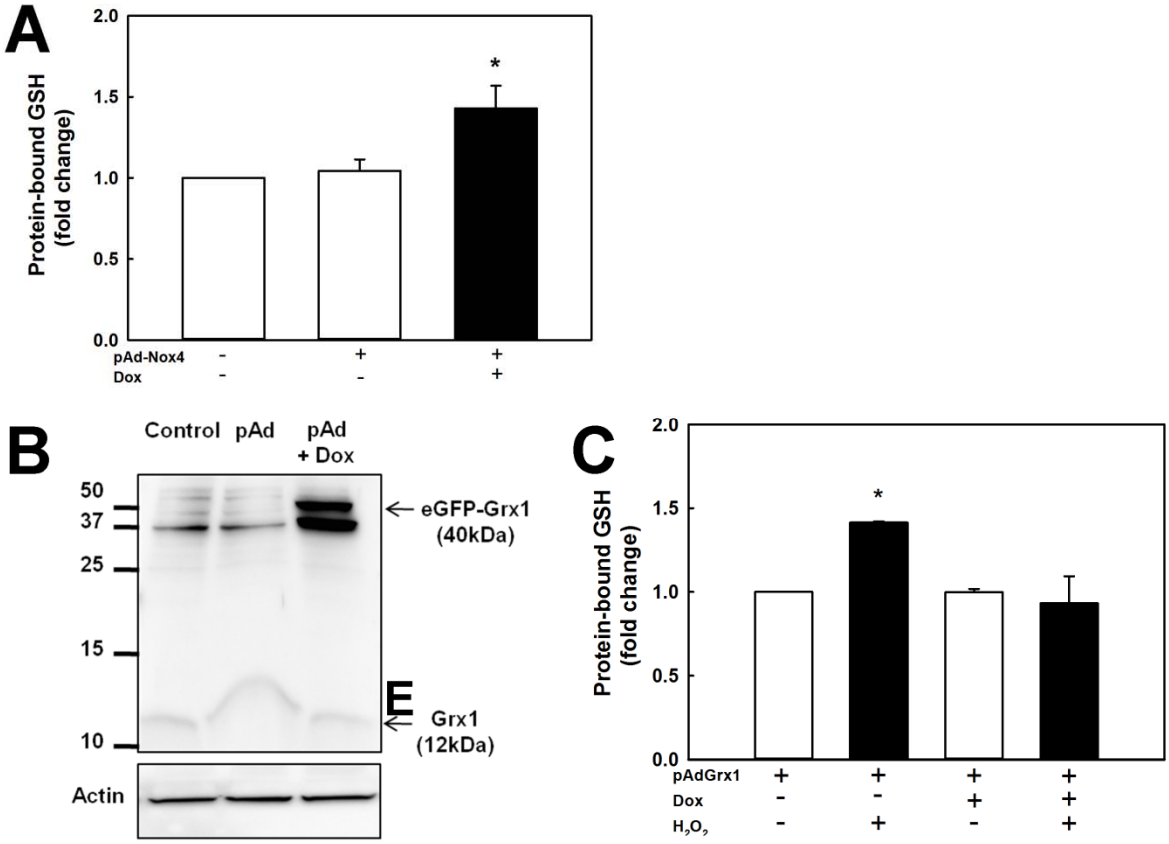
Statistics: Data were analyzed using ANOVA (SPSS 17.0). Data were tested for use of parametric or nonparametric post hoc analysis, and multiple comparisons were performed by using the Least Significant Difference method. All data are presented as mean ± SE of at least 3 independent experiments unless stated otherwise. Results were considered statistically significant at the $P < 0.05$ level.



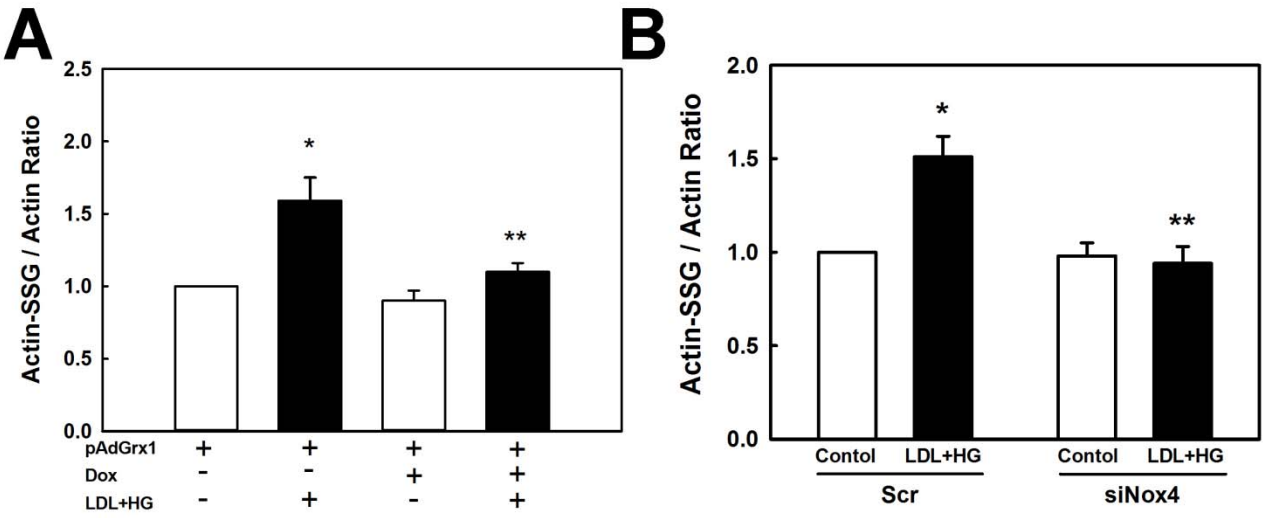
ONLINE FIGURE I: D-Glucose but not L-glucose prime monocytes for MCP-1-induced chemotaxis. THP-1 monocytes were cultured for 20 h in culture medium (RPMI 1640 medium with 10% FBS; Control) or culture medium supplemented with either 20 mM D-glucose (D-Glc) or 20 mM L-glucose (L-Glc). Chemotaxis was induced for 3 h with rMCP-1 (2 nM). Results shown are mean ± SE of 4 independent experiments. *****: $P < 0.05$ versus Control



ONLINE FIGURE II: Nox4 protein expression in human THP-1 monocytes. (A) THP-1 monocytes were cultured for 20 h in culture medium (RPMI 1640 medium with 10% FBS; open symbols) or culture medium supplemented with 100 μg/ml native LDL plus 20 mM D-glucose concentrations (LDL+HG, closed symbols). **(B)** THP-1 monocytes were infected with doxycycline-inducible adenoviruses carrying human Nox4 (pAd). Nox4 expression was induced by adding doxycycline (Dox; 1 μg/ml) to the culture medium for 20 h. **(C)** THP-1 monocytes were transfected with either scrambled siRNA (Scr) or siRNA directed against Nox4 (siNox4) and subsequently cultured for 20 h with medium (Control) or medium supplemented with 100 μg/ml native LDL plus 20 mM D-glucose (LDL+HG). See figure 3 for additional details. **(D)** THP-1 monocytes were transfected with either scrambled siRNA (Scr) or siRNA directed against Nox4 (siNox4), and mRNA levels for Nox4, Nox2 and actin were determined by RT-PCR (NTC: no-template control)



ONLINE FIGURE III: Overexpression of Nox4 promotes whereas Grx1 overexpression protects THP-1 monocytes from metabolic stress-induced protein-*S*-glutathionylation. THP-1 monocytes were infected with doxycycline-inducible adenoviruses (pAd) carrying either Nox4 **(A)** or a Grx1-EGFP fusion construct **(B + C)**. Transgene expression was induced by adding doxycycline (Dox; 1 μg/ml) to the culture medium for 24 h. Cellular levels of protein-*S*-glutathionylation were determined as described under *Methods* Results shown are mean ± SE of 4-5 independent experiments. (**A**) *****: $P < 0.05$ versus infected but uninduced; (**C**): *****: $P < 0.05$ versus infected but uninduced, no H_2O_2).



ONLINE FIGURE IV: Overexpression of Grx1 or knockdown of Nox4 protects THP-1 monocytes from metabolic stress-induced actin-*S*-glutathionylation. (A) THP-1 monocytes were infected with doxycycline-inducible adenoviruses (pAd) carrying a Grx1-EGFP fusion construct. Transgene expression was induced by adding doxycycline (Dox; 1 μg/ml) to the culture medium for 24 h. Monocytes were metabolically stressed by culturing the cells for 20 h with medium supplemented with 100 μg/ml native LDL plus 20 mM D-glucose (LDL+HG). See figure 3 for additional details Cellular levels of actin and *S*-glutathionylated actin were determined as described under *Methods*. Results shown are mean ± SE of 4 independent experiments. *****: $P < 0.01$ versus infected but uninduced and unprimed; ******: $P < 0.05$ versus induced and primed. (**B**): THP-1 monocytes were transfected with either scrambled siRNA (Scr) or siRNA directed against Nox4 (siNox4). Subsequently, monocytes were primed with LDL+HG and cellular levels of actin and *S*-glutathionylated actin were determined as described in A. *****: $P < 0.05$ versus Scr, Control; ******: $P < 0.05$ versus Scr, LDL+HG (n=5).

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