Supplement Material

Supplemental Methods

In vivo and In vitro Analysis of Macrophage Apoptosis

WT and LRP1$^{-/-}$ peritoneal macrophages were collected in PBS 4 days after peritoneal injection of 3% thioglycollate. For *in vivo* analysis of macrophage apoptosis, cells taken directly from the peritoneal cavity were subjected to flow cytometry analysis using AnnexinV/7aaD kit from BD Biosciences, San Diego CA. Macrophages were distinguished from other cell types by co-staining with FITC-conjugated antibody directed to CD11B. For in vitro analysis of apoptosis, WT and LRP1$^{-/-}$ macrophages were seeded in Laboratory-Tek chamber slides (Nalge Nunc International) at 0.5x10^6 cells in DMEM with 10% FBS. The cells were then incubated for 24h in serum-free DMEM alone or containing either lipopolysaccharide (LPS, 50ng/ml) or copper oxidized LDL (50μg protein/ml, Intracel, Frederick, MD). Cell death was then determined by TUNEL (Tdt-mediated dUTP nick end labeling) staining using the in situ cell death detection kit (Roche), according to the manufacturer's instructions. TUNEL-positive cells were quantitated in triplicate chamber slide wells per treatment with cells being counted under light microscope (×400) in 10 fields per well. Apoptosis was also determined by immunohistochemical analysis of activated caspase 3 after WT or LRP1$^{-/-}$ macrophages were incubated for 24h in serum-free DMEM alone. Detection of activated caspase 3 was carried out according to manufacturer's protocol using NucView Caspase Detection kit (Biotium, Hayward CA).

In Vitro Measurement of the Efferocytosis of Apoptotic Macrophages

WT, LRP1$^{-/-}$, or apoE$^{-/-}$ macrophages were made apoptotic and used for in vitro measurement of efferocytosis. Macrophages were collected from the peritoneal cavity four days

post injection with 3% thioglycollate and seeded in 100mm wells at $20x10^6$ cells in DMEM with 10% FBS. The viable macrophages (WT, $apoE^{-/-}$, or $LRP1^{-/-}$) were labeled with 5μM carboxy-fluorescein diacetate, succinimidyl ester (CFDA SE) cell tracer (Molecular Probes, Eugene, OR) in DPBS for 30min at 37ºC in an air atmosphere. The cells were then washed and incubated overnight in serum-free DMEM. To induce apoptosis, the labeled macrophages were incubated with either staurosporine (5μg/mL) for 24h or BAY11-7082 (20μM, Calbiochem) for 2h. Either treatment made >80% of the cells apoptotic as determined by annexinV binding and uptake by WT macrophages was similar using WT cells made apoptotic with the two stimuli (42±4% versus 37±2% positive for uptake. The phagocytes were seeded at $0.5x10^6$ cells/well in 2-well chamber slides. The phagocytes were washed in DPBS, and then $1.5x10^6$ CFDA-SE cell tracer labeled apoptotic cells in DMEM were added to WT, $LRP1^{-/-}$, or $apoE^{-/-}$ efferocytes. After 2h incubation, the phagocytes were washed vigorously four times with PBS to remove noningested apoptotic macrophages. The phagocytes were fixed in 4% paraformaldehyde and counterstained with DAPI for visualization of cell nuclei (Vector Labs, Burlingame CA) and then the efferocytosis of the apoptotic cells was visualized using fluorescence microscopy. CFDA-SE cell tracer positive phagocytes versus total phagocytes were quantitated in triplicate chamber slide wells per treatment with cells being counted in 10 fields per well. The validity of the phagocytosis assay was confirmed by preincubating the WT phagocytes for 24h with the phagocytosis inhibitor, 3-methylchoanthrene,[1] which reduced the uptake of apoptotic WT macrophages by 97%.

Analysis of Efferocytosis in the Peritoneal Cavity

WT macrophages were seeded onto 100 mm tissue culture wells at a density of $20x10^6$ cells in 10%FBS/DMEM. Non-adherent cells were then washed away and the macrophages

were incubated for 48h in 1%FBS/DMEM. The macrophages were then washed three times and labeled with 5μM Vybrant CFDA SE Cell Tracer Green for 30 minutes as described above. The macrophages were then made apoptotic by the addition of 20μM Bay 11-7082 (Calbiochem) in serum-free DMEM for 2h. The CFDA SE -labeled, apoptotic WT macrophages were then harvested by scraping in ice cold PBS, combined with the media supernatant and pooled for centrifugation. Cells were resuspended in PBS, counted and diluted to a concentration of $20x10^6$ per ml. One ml of the WT apoptotic cells was injected into WT or MΦLRP$^{-/-}$ mice that had been injected two days prior with 1ml of 3.0% thioglycollate. Typically, 7 to 10 million cells are recovered in the peritoneal lavage 2 days post injection of WT mice with 1ml of 3% thioglycollate making the apoptotic cell to phagocyte ratio approximately 2 to 3. One hour later the mice were sacrificed and the peritoneal cells harvested by lavage with PBS. Harvested cells were counted, centrifuged and resuspended at concentration of $1x10^6$ per ml in PBS. Three million cells from each mouse were then labeled with 250nM Vybrant Cell Metabolic Assay C_{12}-resazurin solution (Invitrogen) in PBS to label viable cells. Cells were incubated for 15 minutes at 37^o in an open-air incubator. In viable cells, nonfluorescent C_{12}-resazurin is metabolized to red fluorescent C_{12}-resorufin. The cells were then washed with PBS, pelleted by centrifugation and resuspended in 100μl PBS per $1x10^6$ cells. These cells were then labeled with rat anti-mouse CD68-biotin antibody (Serotec) and streptavidin-Alexa Fluor 647 conjugate for 20 minutes before being washed, pelleted and resuspended in 1ml PBS for flow cytometric analysis. Flow cytometry was performed on a 5-laser BD LSRII using FACSDiva 6.0 software (BD Biosciences). Cells positive for CFDA SE + CD68 + C_{12}-resorufin versus CD68 + C_{12}-resorufin only were considered to be phagocytes positive for uptake of apoptotic cells.

Analysis of Atherosclerotic Lesion Apoptosis, Efferocytosis, and Necrosis

Recipient LDLR$^{-/-}$ mice (female, 6-weeks old) were lethally irradiated (9 Gy) using a cesium gamma source and transplanted with 5×10^6 bone marrow cells from female WT or MΦLRP$^{-/-}$ mice through injection into the retro-orbital venous plexus. Four weeks post bone marrow transplantation, the mice were placed on a western-type diet for 16 weeks, and then the extent of atherosclerosis was examined in oil red O-stained cross-sections of the proximal aorta (15 alternate, 10-μm cryosections) using the KS300 imaging system (Kontron Elektronik GmbH). Lesion apoptotic cells were detected in five-micron proximal aortic cryosections by TUNEL after Triton X-100 treatment using the in situ cell death detection kit, TMR red (Roche, Mannheim,Germany) following the manufacturer's instructions. The nuclei were counterstained with Hoechst, and images of the sections were taken using fluorescence microscopy. Five serial sections from each mouse were stained, and the number of apoptotic cells per section were quantitated and normalized to the lesion area as determined by oil red-O staining. We analyzed the efferocytosis in lesions following the procedure as described by Schrijvers et al[2] and as modified by Thorp and colleagues[3]. The same sections that were stained with TMR red TUNEL and Hoechst were also stained for macrophage cytoplasm using a rabbit antimacrophage antibody (AIA31240, Accurate Chemical and Scientific Corp.), goat anti-rabbit biotinylated conjugated secondary antibody, and Alexa Fluor 488 (Molecular Probes, Inc.). The free versus macrophage associated apoptotic cells or bodies were counted in five sections per mouse. Apoptotic cells or bodies were counted as free when they were not associated with or in close proximity to viable macrophages that were detected as Alexa Fluor 488 stained macrophage cytoplasm surrounding a Hoeschst-stained nucleus. Apoptotic cells or bodies that were associated with macrophage cytoplasmic debris, but not in contact or close proximity with viable macrophages were counted as free. To determine lesion necrosis, five serial sections from each

mouse were stained with Harris's hematoxylin and eosin (H&E). Necrosis was quantitated by measuring the H&E negative acellular area in the intima versus total intimal area.

ApoE Secretion and Immunocytochemistry

Macrophages were collected as stated above and $2x10^6$ cells were seeded in DMEM with 10%FBS. Cells were washed twice with cold PBS, and fresh DMEM was added for 24h. Media samples were separated using 10% SDS PAGE. After protein transfer to nitrocellulose, apoE was detected using rabbit anti-serum against mouse apoE (1:1000 dilution) and goat anti-rabbit IgG (1:5000 dilution) conjugated to horseradish peroxidase. Protein was visualized using ECL reagents (Amersham, Piscataway, NJ). For immunocytochemistry studies, cells were seeded at a density of $50x10^5$ per well in DMEM with 10% FBS, and after adherence, the cells were washed and incubated for 24h in serum-free DMEM. For apoE detection, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton X in PBS, and blocked with PBS infused with 10% FBS for 30 min at room temperature. Rabbit anti-serum against mouse apoE (1:1000 dilution) was added to the cells and incubated at 37° C for 1h. The cells were washed 3 times in PBS and incubated with FITC-conjugated goat anti-rabbit for 1h at room temperature. The cells were washed with PBS and cover slips were mounted using Vectashield with DAPI to visualize cell nuclei (Vector Labs, Burlingame CA).

Macrophage Survival and Apoptotic Proteins

Cells were incubated for the indicated times with serum-DMEM alone or containing LPS (50ng/ml) and then lysed in RIPA buffer containing the Sigma protease inhibitor cocktail. The cell proteins were separated using NuPage 10% Bis-Tris gels and then transferred onto nitrocellulose membranes using NuPage transfer buffer for 3h at 26V. Total Akt, phosphorylated Akt (pAkt), and phosphorylated Bad (pBad) were detected using rabbit

polyclonal antibodies to Akt, pAkt$^{(serine\ 473)}$, and pBad$^{(serine\ 136)}$ (Cell Signaling Technology, Inc., Beverly, MA). The protein signal was then detected using goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase and the ECL plus chemiluminescence kit (Amersham, Piscataway, NJ).

Real-time RT PCR and ELISA Analyses

Total RNA was isolated from thioglycollate-elicited peritoneal macrophages using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Relative quantitations of the target mRNA were performed with the ABI Prism 7700 Sequence detection system and normalized with 18S ribosomal RNA and an internal control. Relative mRNA levels for individual genes were determined using TaqMan Assays-on-Demand kits (Applied Biosystems, ABI Foster City, CA). The data were analyzed using comparative C_T methods and are representative of multiple experiments, each with triplicate determinations. Medium cytokine levels were detected using ELISA kits (BD Pharminogen).

Statistical Analysis

In vitro data are expressed as mean ± standard deviation of triplicate determinations. *In vivo* data are expressed as mean ± SEM. Differences between two mean values were determinedby two-tailed Student's t-test, one-way ANOVA (Bonferroni's post test), and Mann-Whitney test. $p<0.05$ was considered to be significant.

References

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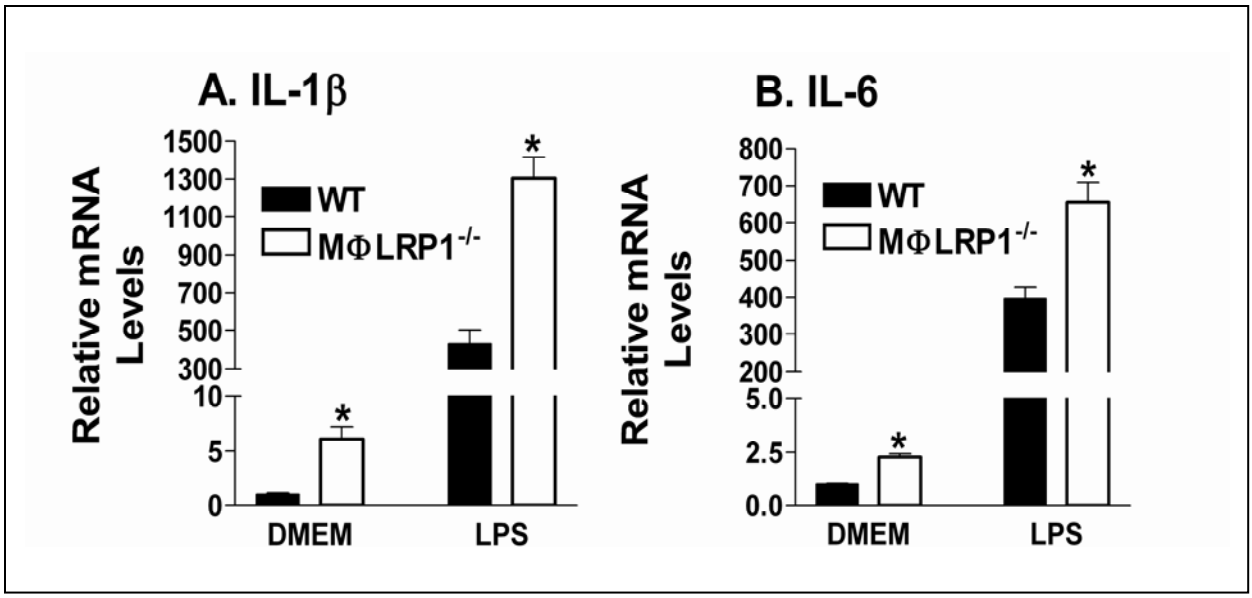

Supplemental Figure I. IL-1β (A) and IL-6 (B) mRNA levels after 6h in serum-free DMEM alone or with LPS (50ng/ml). Data represent 2 experiments. *$p<0.05$, Student's t test.