Plasma HDL levels have a strong inverse relationship to the incidence of heart attack and stroke, but the underlying mechanisms are poorly understood. Results from our previous reports suggest that two ATP-binding cassette transporters ABCA1 and ABCG1 are central to the anti-atherogenic effects of HDL at least in part by mediating macrophage cholesterol efflux and protecting macrophage foam cell formation. However, the past decade has witnessed a dramatic increase in our understanding of the importance of inflammation in all stage of atherosclerotic heart disease and HDL has been reported to have anti-inflammatory properties. In the present study, we demonstrated that cholesterol accumulation in the plasma membrane of *Abcg1*$^{-/-}$ and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ macrophages leads to increased levels and signaling of TLR4 and an increased inflammatory response following exposure to LPS. Significantly, neutrophils became prominent in lesions of *Abcg1*$^{-/-}$ bone marrow transplanted mice after a peripheral inflammatory stimulus most likely reflecting the underlying neutrophilia in these mice as well as the secretion of potent neutrophil chemokines, MIP-1α and MIP-2, by *Abcg1*$^{-/-}$ macrophages. These data suggest that similar changes in human atherosclerotic plaques could be involved in plaque destabilization in subject with low HDL levels after a peripheral inflammatory stimulus and suggest that treatments that raise HDL levels, such as niacin and CETP inhibitors, have the potential to decrease atherosclerosis not only by promoting cholesterol efflux via ABCA1 and ABCG1 but also by suppressing inflammatory and chemokine gene responses in macrophage foam cells.

Online Data Supplement

Methods

Materials. Cell culture media and reagents and heat-inactivated fetal bovine serum (FBS, from GIBCO) were from Invtrogen. Chemical reagents were from Sigma unless speficied below. Human acetyl-LDL (acLDL) and human oxidized-LDL (OxLDL) were purchased from Biomedical technologies. TLR ligands: peptidoglycan, PGN, PolyI:C, LipidA, Gardiquimod, Bacterial CpG-DNA were from Invivogen. Nigericin was from Calbiochem and used as previously described.[1] PAPC was from Aventi Polar Lipids and oxidized by exposure of dry lipid to air as previously described.[2] On-Targetplus Smartpool cRNA oligonucleotides derived from the mouse NR1H3 and NR1H2 target sequences were obtained from Dharmacon and used to induce RNAi to suppress LXRα and LXRβ expression, respectively in thioglycollate-elicited macrophages. The antibodies were obtained as follows : anti-mouse Toll-like receptor 4 was from eBioscience, mouse anti-Nucleophosmin from was Zymed Laboratories Inc., mouse anti-p65 NF-κB from Cell Signaling Technology.

Histological analysis of proximal aortas. Proximal aortas were serially paraffin-sectioned and stained with hematoxvlin and eosin as previously described.[3] Aortic lesion size of each animals was obtained by average lesion areas in five sections from the same mouse. Mac-3 and MCA771G antibodies (BD pharmingen) were used to stain macrophages and neutrophils in paraffin-sections, respectively.

White blood cell counts. Whole blood was analyzed using a hematology cell counter (Ac-Tdiff, Beckman Coulter) and neutrophils were differentiated using a commercial kit (Wright-Giemsa Stain, Sigma).

Macrophage harvest and treatments. Peritoneal macrophage cells were harvested from mixed C57BL/6 x DBA WT, *Abcg1*$^{-/-}$, *Abca1*$^{-/-}$ and *Abca1*$^{-/-}$*Abcg1*$^{-/-}$ littermate mice 3 days after receiving an i.p injection of thioglycollate and plated in 10% FBS in DMEM media as previously described.[4] For some experiments, C57BL/6 WT and *Abcg1*$^{-/-}$ mice were used as described in the figure legends. Myd88-/- and Myd88-/-Trif-/- on the C57BL/6 background were from Dr. Katherine Fitzgerald (University of Massachusetts). Adherent cells consisting of macrophages were used for experiment as described in the figure legend. Bone-marrow-derived macrophages were isolated and cultured in 10% FBS in DMEM media supplemented with macrophage-colony

stimulating factor for 5-10 days before the experiment.[5] RNA interference to suppress ABCG1 expression in mouse macrophages was performed as previously described.[6] Where indicated, cells were incubated with 50ng/mL lipopolysaccharides (LPS, Escherichia coli 0111:B4, Sigma) in 0.2% BSA DMEM and in presence of 5mM ATP for bone-marrow derived macrophages. Cyclodextrin (CD, Sigma) and CD-cholesterol (2.5:1 molar ratio) media was prepared as decribed.[7] Briefly, cellular cholesterol was depleted by 5mM CD for 30 min before LPS treatment and cholesterol loading was performed by CD-chol for 3 hours. Filipin (Sigma) was pre-incubated at 3μg/mL, 30 min before addition of LPS to the cells and during the 3h of LPS treatment

Confocal microscopy. Macrophages were cultured on coverslips in 0.2% BSA in DMEM media (basal state) or in presence of 50μg/mL AcLDL plus 3μmol/L LXR agonist (TO901317) for 24 hours (loaded state). Lipid rafts were labelled in live cells using a commercial kit (Vybrant Lipid Raft Labeling, Molecular Probes) and then, fixed with 4% paraformaldehyde (Sigma) for 15 min at 4°C. The coverslips were mounted using Mowiol (Calbiochem) and visualized with a Axioskop 2 FS MOT upright confocal microscope (Zeiss). Images were obtained by implementing z-scanning and analyzed using the ImageJ software.

Sterol mass quantification. Sterols were determined by gas-liquid chromatography or gas chromatography-mass spectrometry as previously described.[8,9]

Flow cytometry analysis. Thioglycollate-elicited cells were cultured for 1h in suspension in presence or absence of 50ng/mL LPS. Macrophage surface expression of TLR4 and TLR4/MD2 complex was detected by flow cytometry of live cells stained with Alexa Fluor 488 anti-mouse TLR4 (UT41, eBioscience) or FITC anti-mouse TLR4/MD2 complex (MTS510, Stressgen) plus Phycoerythrin (PE) anti-mouse Mac-3 (eBioscience). The two anti-TLR4 antibodies differ insofar as the UT41 antibody recognizes TLR4, whereas the MTS510 recognizes the TLR4/MD2 complex. 20,000 cells/condition were analyzed in a FACScan (Becton Dickinson).

RNA analysis. Total RNA extraction, cDNA synthesis and real-time PCR was performed as described previously.[5] β-Actin RNA expression was used to account for variability in the initial quantities of mRNA.

Secretion analysis. Cytokines and chemokines were measured enzyme-linked immunoabsorbent assay (ELISA) (R&D Systems and RayBiotech, Inc.).

Cellular extracts. Total protein extracts were prepared from cells for caspase-1 immunobloting as previously described.[10] Nuclear and endoplasmic reticulum membrane fractionation were performed as previously described.[11,12] Briefly, nuclear and cytosol fractions were analyzed by SDS-PAGE and immunobloting for organelle markers, TLR4 and p65 NF-κB. Endoplasmic reticulum membrane were prepared by sucrose step gradient and centrifuged at 10,000g. Five fractions were collected and the pellet enriched in endoplasmic reticulum was resuspended in 0.5mL of buffer before lipid extraction. Free cholesterol was determined by gas-liquid chromatography.

Western blot analysis. Cell extracts were electrophoresed on 4-20% gradient SDS-PAGE gels and transferred to 0.22-μm nitrocellulose membranes. The membrane was blocked in Tris-buffered saline, 0.1% Tween20 containing 5%(w/v) nonfat milk (TBST-nfm) at room temperature (RT) for 1h and then incubated with the primary antibody in TBST-nfm at RT for 4h, followed by incubation with the appropriate secondary antibody coupled to horseradish peroxidase. Proteins were detected by ECL chemiluminescence (Pierce).

Statistical analysis. Data are given as means ± S.E.M. Comparison of mean values between groups was evaluated by two-tailed parametric student's t test, non-parametric Mann-Whitney U test (2-group lesion area and adventitial neutrophils, clotting parameter measurements), or by one-way analysis of variance (ANOVA, 4-group comparisons) with a Bonferroni multiple comparison post-test (GraphPad software, San Diego, CA). When appropriate, square root or log transformation was applied to approximate a normal distribution for ANOVA. Differences were considered significant at $P < 0.05$.

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Fig. S1

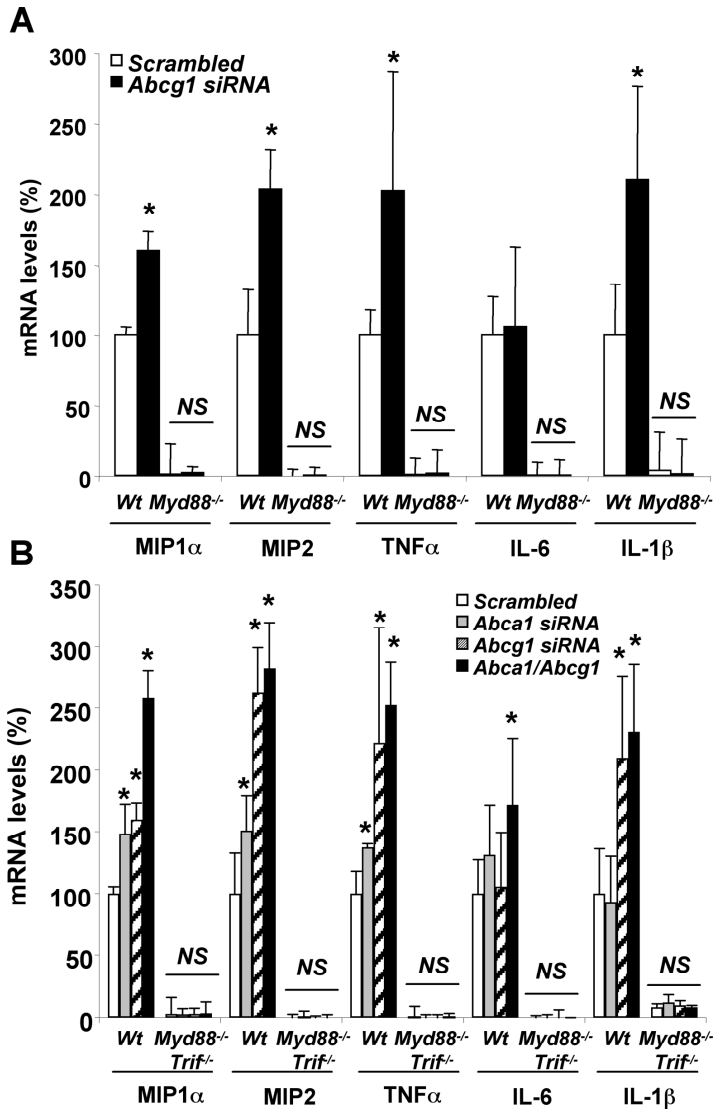

Fig. S1. Increased inflammatory response in macrophages lacking Abca1 and Abcg1 requires Myd88-Trif signaling. Bone marrow derived macrophages from *Myd88-/-* (A) and *Myd88-/-,Trif-/-* (B) mice were treated with either Abca1, *Abcg1* or Abca1 and Abcg1 siRNA as described in the figures before 50ng/mL LPS treatment for 4h in 0.2% BSA DMEM. Expression of mRNA was normalized to β-actin and expressed as percentage over LPS-treated WT macrophages. Results are mean ± SEM. **P*< 0.05 vs. WT.

Fig. S2

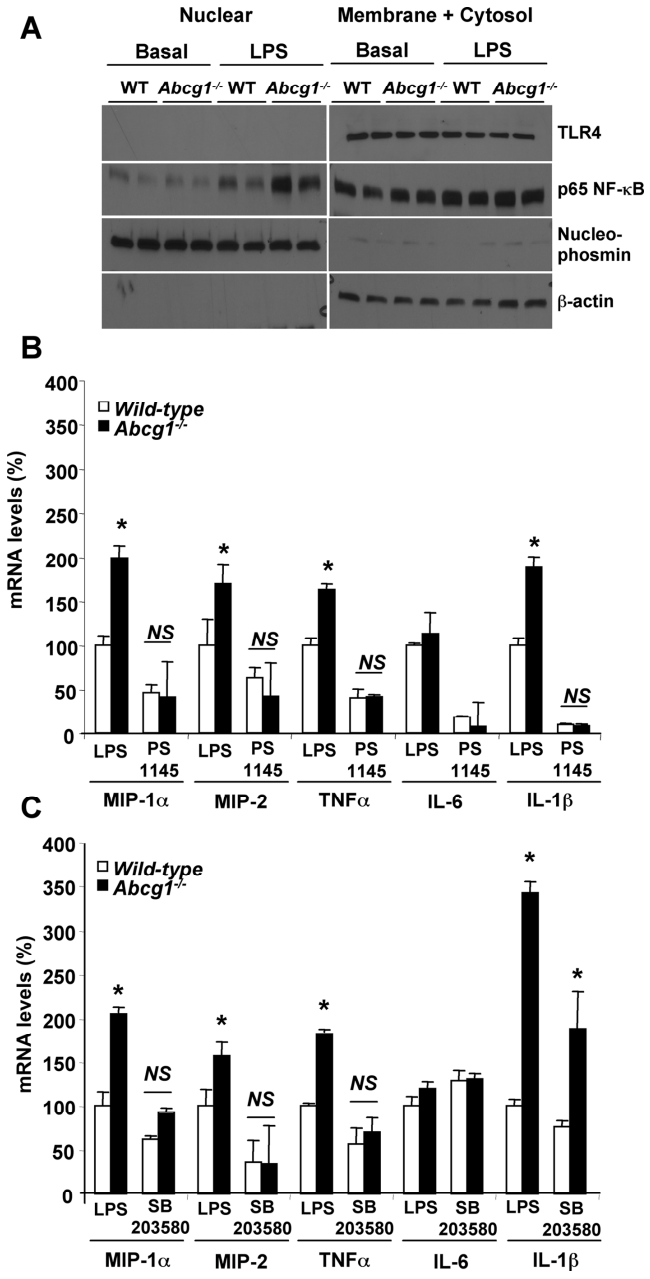

Fig. S2. Enhanced NF-κB activity in *Abcg1-/-* macrophages. Thioglycollate-elicited macrophages from C57BL/6 WT and Abcg1-/- mice were subjected to nuclear fractionation and analyzed for TLR4, p65-NFκB, nucleophasmin and β-actin expression (A). Cells were treated with 50ng/mL LPS for 4h in the presence or absence of an NFκB inhibitor (PS1145) (B) or a p38 MAPK inhibitor (SB203580) (C). Expression of mRNA was normalized to β-actin and expressed as percentage over LPS-treated WT macrophages. Results are mean ± SEM. **P*< 0.05 vs. WT.

Fig. S3

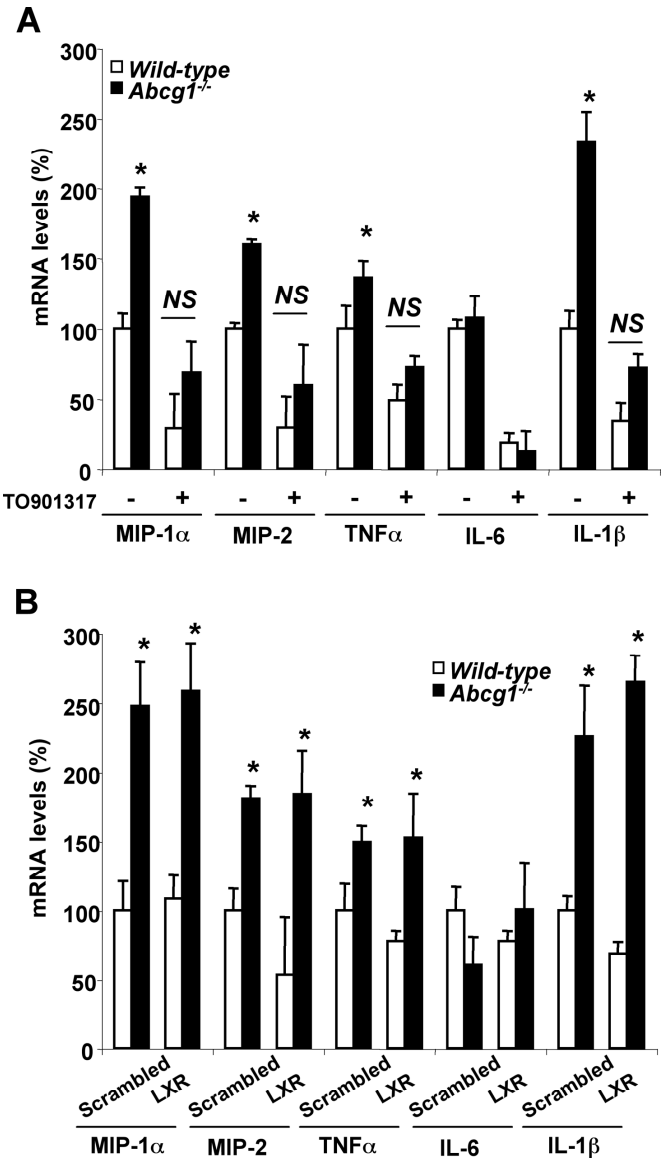

Fig. S3. Increased inflammatory response in *Abcg1*-/- macrophages does not involve LXR transrepresion mechanism. Thioglycollate-elicited macrophages from C57BL/6 WT and Abcg1-/- mice were treated overnight with an LXR agonist (3μmol/L TO901317) (A) or with scrambled or LXRαβ siRNAs for 32 hours (B) before 50ng/mL LPS treatment for 4h. Expression of mRNA was normalized to β-actin and expressed as percentage over LPS-treated WT macrophages. Results are means ± SEM. **P*< 0.05 vs. WT.

Fig. S4

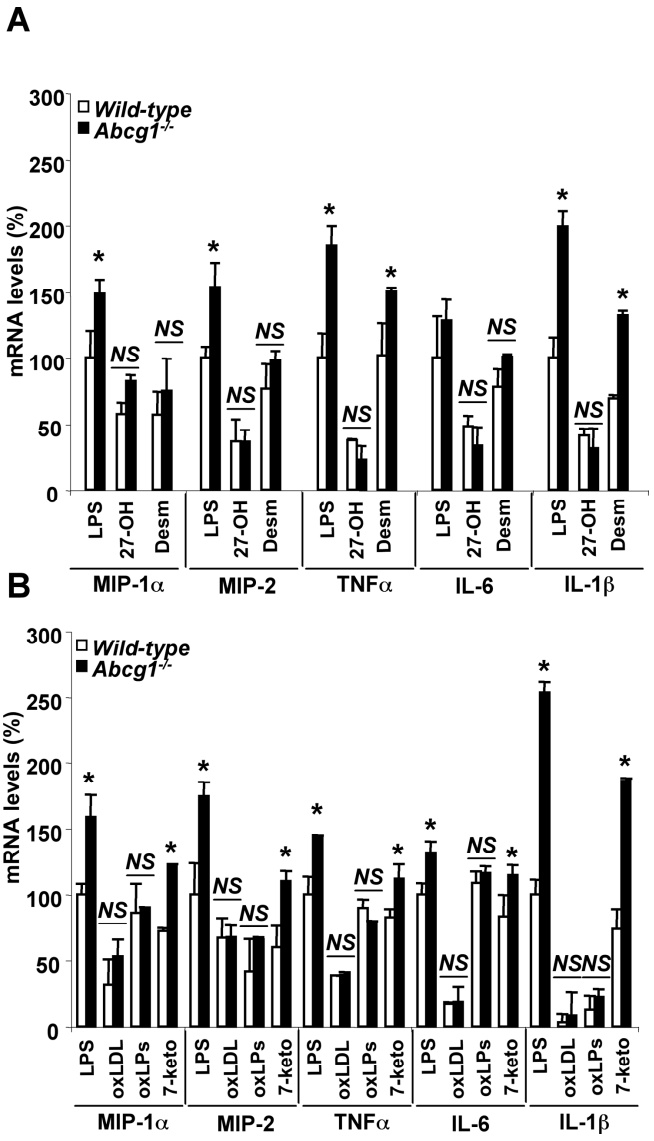

Fig. S4. Increased inflammatory response in *Abc* deficient macrophages does not involve specific LXR ligand accumulation or oxidized phospholipids. Thioglycollate-elicited macrophages from C57BL/6 WT and Abcg1-/- mice were treated overnight with 10μM 27-OH cholesterol or 10μM desmosterol before 50ng/mL LPS treatment for 4h (A). Cells were also loaded overnight with 50μg/mL oxLDL, 25μg/mL oxidized-PAPC or 10μM 7-ketocholesterol before LPS treatment (B). Expression of mRNA was normalized to β-actin and expressed as percentage over LPS-treated WT macrophages. Results are means ± SEM. **P*< 0.05 vs. WT.

Fig. S5

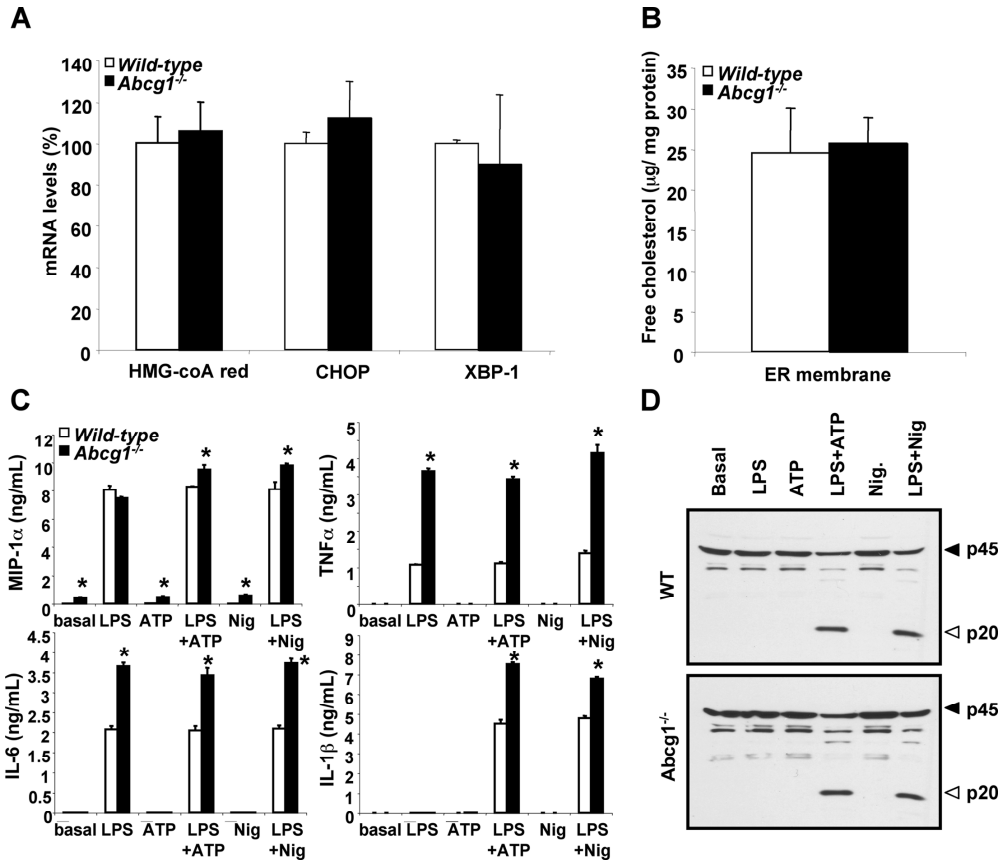

Fig. S5. Increased inflammatory response in *Abcg1*-/- macrophages does not involve ER stress or inflammasome. ER stress markers (HMG-coAred, CHOP and XBP-1) mRNA expression was analyzed in C57BL/6 WT and Abcg1-/- macrophages cultured in 10% FBS DMEM media (A). Thioglycollate-elicited macrophages from WT and Abcg1-/- mice were subjected to ER-membranes fractionation and cholesterol content was analyzed by gas chromatography (B). Production of inflammatory cytokines (MIP-1α, TNFα, IL-6 and IL-1β) (C) and immunoblot of caspase-1 (D) from C57BL/6 x DBA WT and Abcg1-/- bone marrow-derived macrophages stimulated with 50ng/mL LPS for 4 hours and then pulsed with medium, 5mM ATP or 20μM nigericin for 30 min. Secretion levels were determined 3h post-induction and expressed as ng/mL (C). Results are means ± SEM. **P*< 0.05 vs. WT. Arrows denote procaspase-1 (p45) and its processed large submit (p20) (D).