MEK1/2 inhibitors activate macrophage ABCG1 expression and reverse cholesterol transport—an anti-atherogenic function of ERK1/2 inhibition

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Abstract

Expression of ATP-binding cassette transporter G1 (ABCG1), a molecule facilitating cholesterol efflux to HDL, is activated by liver X receptor (LXR). In this study, we investigated if inhibition of ERK1/2 can activate macrophage ABCG1 expression and functions. MEK1/2 inhibitors, PD98059 and U0126, increased ABCG1 mRNA and protein expression, and activated the natural ABCG1 promoter but not the promoter with the LXR responsive element (LXRE) deletion. Inhibition of ABCG1 expression by ABCG1 siRNA did enhance the formation of macrophage/foam cells and it attenuated the inhibitory effect of MEK1/2 inhibitors on foam cell formation. MEK1/2 inhibitors activated macrophage cholesterol efflux to HDL *in vitro*, and they enhanced reverse cholesterol transport (RCT) *in vivo*. ApoE deficient (apoE^{-/-}) mice receiving U0126 treatment had reduced sinus lesions in the aortic root which was associated with activated macrophage ABCG1 expression in the lesion areas. MEK1/2 inhibitors coordinated the RXR agonist, but not the LXR agonist, to induce ABCG1 expression. Furthermore, induction of ABCG1 expression by MEK1/2 inhibitors was associated with activation of SIRT1, a positive regulator of LXR activity, and inactivation of SULT2B1 and RIP140, two negative regulators of LXR activity. Taken together, our study suggests that MEK1/2 inhibitors activate macrophage ABCG1 expression/RCT, and inhibit foam cell formation and lesion development by multiple mechanisms, supporting the concept that ERK1/2 inhibition is anti-atherogenic.

Keywords: ABCG1; atherosclerosis; LXR; MEK1/2; RCT; SIRT1

Introduction

Atherosclerosis is one of major causes for coronary heart diseases (CHD), and a chronic pathological process with disorders in lipid metabolism and inflammation¹. Recruitment of monocytes, subsequent monocyte/macrophage differentiation in arteries and formation of lipid-laden macrophage/foam cells are critical steps in the development of lesions². Macrophage cholesterol homeostasis depends on cholesterol uptake and free cholesterol efflux which are mediated by multiple molecules^{3, 4}. The un-controlled cholesterol uptake by scavenger receptors, such as CD36, facilitates foam cell formation. In contrast, foam cell formation is inhibited by cholesterol efflux from macrophages. Macrophage cholesterol efflux and reverse cholesterol transport (RCT) can substantially reduce atherosclerosis. The ATP-binding cassette transporter G1 (ABCG1), a member of the ABC transporter family, mediates cholesterol efflux from macrophage/foam cells to the extracellular cholesterol acceptor, high-density lipoprotein (HDL)^{5, 6}. Deficiency of ABCG1 expression can disrupt lipid homeostasis and enhance lesion development in LDL receptor deficient (LDLR^{-/-}) mice⁷. The combined deficiency of ABCG1 and ATP-binding cassette transporter A1 (ABCA1) expression can promote foam cell formation and atherosclerosis in both wild type and LDLR^{-/-} mice^{8, 9}. However, some variable effects of ABCG1 on cholesterol efflux or atherosclerosis have also been reported. For example, in lipid-loaded THP-1 macrophages, inhibition of ABCG1 expression by siRNA has little effect on LXR ligand-activated cholesterol efflux¹⁰. Deficiency of ABCG1 expression increases lesions at the early stage while decreasing lesion at the advanced stage of

atherosclerosis in LDLR^{-/-} mice¹¹.

Similar to ABCA1, ABCG1 expression is transcriptionally activated by the liver X receptor (LXR), a ligand-activated transcription factor¹². Several oxysterols, such as 24S-hydroxycholesterol and 25-hydroxycholesterol, serve as endogenous LXR ligands¹³. Meanwhile, some synthetic ligands, such as T0901317 and GW3965, have been demonstrated to potentiate LXR activity¹⁴. LXR activation by its ligand can lead to formation of a heterodimer of LXR with another nuclear protein, retinoid X receptor (RXR). The complex of LXR/RXR binds to the LXR responsive element (LXRE) in the promoter of LXR target genes thereby activating their transcription¹². The LXRE is also called DR4 because the six conserved nucleotides are repeated in this motif (AGGTCAN₄AGGTCA) separated by any four nucleotides.

Extracellular signal-regulated kinases 1/2 (ERK1/2) can be activated by different growth stimuli through a kinase cascade in which both Thr/Tyr residues in ERK1/2 are phosphorylated by MAP kinase kinases 1/2 (MEK1/2)¹⁵. ERK1/2 activity is involved in a variety of cellular activities, such as differentiation, proliferation, immune response and metabolism¹⁶. In the vascular system, ERK1/2 has been determined to be highly expressed and activated in lesion areas of cholesterol-fed rabbits¹⁷. We previously reported that inhibition of ERK1/2 by MEK1/2 inhibitors synergized LXR ligand-induced ABCA1 expression and cholesterol efflux to apoAI, another extracellular cholesterol acceptor¹⁸. Furthermore, we showed that the combination of MEK1/2 inhibitor (U0126) and LXR ligand (T0901317) can synergistically reduce atherosclerosis in apoE deficient (apoE^{-/-}) mice without the adverse effects of hepatic

lipogenesis and hypertriglyceridemia which is induced by T0901317 alone¹⁹. These findings indicate that ERK1/2 activity is involved in the development of atherosclerosis, while MEK1/2 inhibitors may have multiple anti-atherogenic functions. Therefore, in this study we investigated the effect of MEK1/2 inhibitors on macrophage ABCG1 expression and functions.

Material and methods

Materials

PD98059 and U0126 were purchased from LC laboratories (Woburn, MA). Resveratrol, nicotinamide and 9-*cis*-retinoic acid (9-*cis*-RA) were purchased from Sigma-Aldrich (St Louis, MO). Both low-density lipoprotein (LDL) and HDL were purchased from Athens Research & Technology, Inc (Athens, GA). Acetylated LDL (AcLDL) was prepared as described²⁰. Anti-ABCG1 (Cat #: 13578-1-AP) and LXR rabbit polyclonal antibodies were purchased from Proteintech Group (Chicago, IL). Anti-SIRT1 rabbit polyclonal and anti-monocyte/macrophage marker (MOMA2) rat monoclonal antibodies, and siRNA against ABCG1 (si-ABCG1) and SIRT1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture and determination of macrophage cholesterol efflux to HDL

RAW264.7 murine macrophage cell line and THP-1 human monocytic cell line were purchased from ATCC (Rockville, MD), and cultured in complete RPMI medium containing 10% fetal calf serum, 50 µg/ml penicillin and streptomycin, and 2 mM glutamine.

To induce monocyte/macrophage differentiation, THP-1 monocytes were adjusted to a density of $\sim 2.5 \times 10^5$ cells/cm² in 6-well plates and treated with 200 nM phorbol 12-myristate 13-acetate (PMA) overnight in complete RPMI medium. After removal of PMA, the adhesive cells (THP-1/PMA macrophages) were washed twice with PBS, and continued to be cultured in complete RPMI medium for another 2 days. RAW264.7 macrophages at $\sim 90\%$ confluence or THP-1/PMA macrophages received treatment in serum-free medium.

To collect peritoneal macrophages, C57BL/6 wild type or apoE^{-/-} mice (both are ~8 weeks old) were i.p. injected with 3 ml 4% thioglycollate solution. About 5 days later, peritoneal macrophages were collected from mouse abdomen by lavage with PBS twice, and cultured in complete RPMI medium for 1 h followed by removal of the floating cells. The adhesive cells, which were determined as macrophages by immunofluorescent staining with anti-MOMA2 antibody, were cultured in complete RPMI medium for another 2 days before treatment in serum-free medium.

To determine cholesterol efflux to HDL, peritoneal macrophages in 12-well plates were labeled in serum-free medium containing AcLDL (50 µg/ml) and 150 nCi/ml [³H]-cholesterol for 24 h followed by treatment. Cells were then washed twice with PBS and incubated for 1 h in serum-free medium, switched to serum-free medium or medium containing HDL. After 5 h incubation at 37°C, the medium from each well was collected for determination of radioactivity in the supernatant. The remaining cells in each well were lysed by addition of 0.2 N NaOH followed by determination of cellular protein which was used to normalize cholesterol efflux. The radioactivity in medium and cellular lysate were determined to ensure that the labeling efficiency is similar for all groups. The radioactivity in the group incubated with no HDL added medium was considered as background, and the background was subtracted from those groups incubated with “HDL added medium”. Therefore, the net results after subtraction are the cholesterol efflux to HDL, and expressed as CPM/µg cellular protein.

Western blot and immunofluorescent staining

Cells were washed with PBS and lysed in an ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 50 µg/ml aprotinin/leupeptin). After

extracted, total cellular protein was used to determine ABCG1 and SIRT1 protein expression by Western blot as described²⁰. ABCG1 protein was also determined by immunofluorescent staining as follows: peritoneal macrophages cultured on cover slips were fixed with 4% paraformaldehyde (PFA) for 30 min, washed with PBS for 10 min, and blocked with 2% BSA for 2 h at room temperature (RT). Cells were then incubated with anti-ABCG1 antibody overnight at 4°C followed by incubation with TRITC-conjugated goat anti-rabbit IgG for 2 h at RT. The slips were then stained with DAPI solution to detect nucleus followed by observation and photograph with a fluorescence microscope (Leica, Wetzlar, Germany).

Real time RT-PCR and Northern blot analysis

After treatment, cells were used to extract total RNA²⁰. Expression of ABCG1, SIRT1, SULT2B1 and RIP140 mRNA was determined by real time RT-PCR as follows: 1 µg total RNA was used to synthesize cDNA with the RT kit purchased from New England Biolabs (Ipswich, MA). Real time PCR was performed using a SYBR green PCR master mix purchased from Bio-Rad with the primers listed in Table 1. Expression of SIRT1, ABCG1, SULT2B1 or RIP140 mRNA was normalized by GAPDH mRNA in the corresponding sample, respectively.

Expression of ABCG1 mRNA in RAW264.7 macrophages in response to MEK1/2 inhibitors treatment (Figs. 2A, 2B) was determined by Northern blot as described²⁰. The probe DNA for ABCG1 was generated by RT-PCR with the following primers: forward, 5'-GCTGGGAAGTCCCACTC-3'; backward, 5'-GATACGGCACGAGATTGG-3'.

Inhibition of macrophage ABCG1 expression by siRNA and determination of foam cell

formation

RAW264.7 macrophages and peritoneal macrophages isolated from apoE^{-/-} mice were plated in 6-well plates (for the transfection efficiency assay) or on coated 12-mm glass slides in 24-well plates (for Oil Red O staining), and transfected with scrambled siRNA or ABCG1 siRNA using Lipofectamine™ 2000 (Invitrogen) in RPMI 1640 medium. After 12 h transfection, the same volume of medium was added to cells, and the transfection was continued for another 12 h. The transfected cells were then switched into complete medium for another 48 h. Cells in 6-well plates were used to determine ABCG1 expression by real time RT-PCR or Western blot. Cells in 24-well plates received the indicated treatment for 8 h followed by addition of 25 µg/ml AcLDL and incubation for another 8 h. Cells were then washed with PBS, and incubated in serum-free RPMI 1640 medium containing 15 µg/ml HDL for another 8 h. Cells were then fixed in 4% PFA for 30 min, washed twice with PBS for 5 min, and stained with Oil Red O solution (0.3% Oil Red O in 60% isopropanol) for 60 min at RT followed by washing twice with water. Cells were then counterstained with haematoxylin solution for 30 s, kept in water for 5 min, and photographed. To quantify the accumulated Oil Red O dye within macrophages, cells in 12-well plates were washed three times with PBS after staining, and then the same volume of isopropanol was added to each well to extract dye by incubating at RT with shaking for 15 min. The extraction solution was collected and quickly spun for 1 min followed by determination of absorbance at 510 nm. Cells remaining in wells were lysed and cellular lysate was used to determine protein content which was used to normalize the absorbance of extraction solution. The absorbance in unit protein in control samples were defined as 1.

Plasmid preparation and detection of ABCG1 promoter activity

Mouse ABCG1 promoter (from -724 to +68, the translational start site is from +122) was constructed by PCR with mouse genomic DNA and the following primers: forward, 5'-GGCCGGTACCACTTGCCTGCCCCTTTCTAGGAGAG-3'; backward, 5'-TATCCTCGAGACGAGGTTGCAGGAACGAGGCTGC-3'. After sequence was confirmed, the PCR product was digested by KpnI and XhoI followed by ligation with pGL4.10 luciferase reporter vector (Promega, Madison, WI), transformed, and amplified (pABCG1). The ABCG1 promoter with a complete deletion of the LXRE or DR4 [AGTCGGAACCCGcgcagaGCGCcgcggaGGAGCAGGAGCAAGC, the italic letters is the LXRE (from +13 to +28) in mouse ABCG1 gene, and the underlined small letters are the repeated nucleotides in this LXRE] was constructed with the pABCG1 plasmid and the following primers: forward, 5'-CGCAGTCGGAACCCGGGAGCAGGAGCAAGC-3'; backward, 5'-GCTTGCTCCTGCTCCCGGGTTCCGACTGCG-3'. The cDNA encoding mouse LXR β was generated by RT-PCR with total cellular RNA isolated from macrophages and the following primers: forward, 5'-TAGCCTCGAGCATGTCTTCCCCCACAAGTTC-3'; backward, 5'-CGACAAGCTTCTACTCGTGACATCCCAGATC-3'. After sequence was confirmed, the PCR product was digested by XhoI and HindIII followed by subcloning into an expression vector, pEGFP-C2 or C2, and named as pEGFP-LXR β or LXR β .

293T cells (ATCC) were cultured in 24-well plates with complete DMEM medium. Cells at ~95% confluence were transfected with DNA for the ABCG1 promoter, LXR β expression vector and *Renilla* (for internal normalization) using LipoD293 (SignaGen, Rockville, MD). After 24 h of transfection plus treatment, cells were lysed and cellular lysate was used to determine activity of Firefly and *Renilla* luciferases using the Dual-Luciferase Reporter Assay System (Promega).

Determination of LXR-DNA binding activity by ChIP assay

After treatment, cells were washed with ice-cold PBS, and cross-linked by addition of formaldehyde and sonication in a lysis buffer [50 mM Hepes-KOH (pH 7.5), 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS and protease inhibitors aprotinin/leupeptin] to fragment DNA into an average size of 500-1,000 bp. The input PCR was conducted with DNA extracted from the sonicated chromatin after reversal of the cross-linking. Immunoprecipitation was conducted with the same amount of chromatin from each sample based on the input and rabbit anti-LXR polyclonal antibody, followed by PCR. The primers for the ChIP assay were: forward, 5'-CCACTGGTTCGTCTAGGGTT-3'; and backward, 5'-GCGAAGGATGTGCGACTCA-3'.

In vivo study

The protocols for *in vivo* study with mice were approved by the Ethics Committee of Nankai University and conform to the Guide for the Care and Use of Laboratory Animals published by NIH.

ApoE^{-/-} mice were randomly divided into two groups (15 mice/group) and fed an atherogenic diet (0.5% cholesterol and 21% fat in mass) or an atherogenic diet containing U0126 (3 mg/100 g food) for 16 weeks. After treatment, animals were anesthetized and euthanized in a CO₂ chamber. The 5 μm frozen sections of aortic root were collected. The lipid accumulation in lesion areas was determined by Oil Red O staining. ABCG1 protein expression and localization in lesion areas were determined by immunofluorescent staining with anti-ABCG1 and anti-MOMA2 antibodies. The sections were also stained with DAPI solution.

To determine reverse cholesterol transport (RCT) *in vivo*, peritoneal macrophages

in suspension were radiolabeled in serum-free RPMI medium containing 50 $\mu\text{g/ml}$ AcLDL and 2 $\mu\text{Ci/ml}$ [^3H]-cholesterol for 24 h. Cells were then washed twice with medium, equilibrated in medium containing 0.2% BSA for 4 h. After spun down, the cells were re-suspended with serum-free RPMI medium immediately before use. C57BL/6 mice in two groups (5 mice/group) were fed normal chow and chow containing U0126 (3 mg/100 g food), respectively, for 2 weeks. Each mouse was then i.p. injected with the radiolabeled macrophages ($\sim 2 \times 10^6$ cells containing 1.2×10^6 cpm) followed by collection of fecal samples in 8 h intervals. After 48 h of cell injection, mice were anesthetized and euthanized in a CO_2 chamber followed by collection of blood (for serum isolation), peritoneal macrophages, and a piece of liver. After lipid extraction, radioactivity in samples of feces, liver and serum was determined with a liquid scintillation counting. The values are expressed as a percent of total injected [^3H]-cholesterol.

Data analysis

All experiments were repeated at least three times, and the representative results are presented. Values are represented as mean $\pm$ SEM. All the data were initially subjected to a normal distribution analysis with SPSS software (1-sample K-S of non-parametric test). The *in vivo* RCT data in Figure 4B were obtained by repeated determination of samples ($n=3$). All other data were analyzed by a parametric statistics, post-hoc test of one-way ANOVA if the data was in a normal distribution. A significant difference was considered if $P < 0.05$ ($n \geq 3$).

Results

MEK1/2 inhibitors activate macrophage ABCG1 protein expression

We initially treated RAW264.7 macrophages with MEK1/2 inhibitors, PD98059 and U0126, overnight and determined ABCG1 protein expression by Western blot. Fig. 1A demonstrates that both PD98059 and U0126 induced ABCG1 expression at different concentrations with the maximum induction at 10 μ M (PD98059) and 0.5 μ M (U0126), respectively. The time course study (Fig. 1B) indicates that induction of ABCG1 expression can last for 24 h after treatment with a faster induction by U0126 than PD98059.

Treatment of peritoneal macrophages with PD98059 and U0126 also induced ABCG1 expression at a degree which is comparable to an LXR ligand, T0901317 (top panel, Fig. 1C). The immunofluorescent staining further confirms the induction of ABCG1 expression by MEK1/2 inhibitors (bottom panel, Fig. 1C). Similar to RAW264.7 cells, both PD98059 and U0126 induced primary macrophage ABCG1 expression quickly (Fig. 1D).

We also treated THP-1/PMA macrophages with PD98059 and U0126, and determined that MEK1/2 inhibitors induced THP-1/PMA macrophage ABCG1 expression (Fig. 1E) indicating that the induction of macrophage ABCG1 expression by MEK1/2 inhibitors is not species dependent.

MEK1/2 inhibitors activate ABCG1 transcription

We initially determined the effect of MEK1/2 inhibitors on RAW264.7 macrophage

ABCG1 mRNA expression by Northern blot. Consistent with protein expression (Figs. 1A, 1B), the results in both concentration (Fig. 2A) and time course (Fig. 2B) studies show that PD98059 and U0126 increased ABCG1 mRNA expression in RAW264.7 cells. We also determined ABCG1 mRNA expression in peritoneal macrophages in response to MEK1/2 inhibitor treatment by real time RT-PCR, and obtained similar results to RAW264.7 cells which indicate that MEK1/2 inhibitors activated primary macrophage ABCG1 mRNA expression in a concentration and time dependent manner (Figs. 2C, 2D).

ABCG1 transcription is activated by LXR owing to the existence of an LXRE in its promoter. As shown in the left half of Fig. 2E, compared to the ABCG1 promoter alone, high expressing LXR increased ABCG1 promoter activity. Both PD98059 and U0126 further enhanced ABCG1 promoter activity.

By completing a sequence alignment analysis, we identified a putative LXRE motif in the proximal region of mouse ABCG1 promoter. The sequence of this LXRE is CGCAGAgcgcCGCGGA (the underlined letters are repeated nucleotides), and it locates from +13 to +28 (the translational start site is from +122). To determine the role of this LXRE in MEK1/2 inhibitor activated ABCG1 transcription, we constructed an ABCG1 promoter with complete deletion of this LXRE or DR4 (pABCG1-DR4del). Compared to the natural ABCG1 promoter, Fig. 2E (right half) demonstrates that deletion of this LXRE reduced ABCG1 promoter activity indicating its importance in ABCG1 transcription. Furthermore, we determined that deletion of this LXRE abolished the MEK1/2 inhibitor or T0901317 induced ABCG1 promoter activity.

To further define the role of this LXRE in MEK1/2 inhibitor activated ABCG1 transcription, we determined the binding of LXR protein with this motif in response to MEK1/2 inhibitor and T0901317 treatment by a ChIP assay. The results in Fig. 2F demonstrate that T0901317 and MEK1/2 inhibitors increased the interaction between LXR protein and the LXRE. Taken together, the above findings (Figs. 2E, 2F) suggest that MEK1/2 inhibitors activate ABCG1 transcription in an LXRE-dependent manner.

MEK1/2 inhibitors inhibit macrophage lipid accumulation, increase macrophage cholesterol efflux to HDL in vitro and RCT in vivo, and inhibit sinus lesions

To define the biological functions of increased ABCG1 expression by MEK1/2 inhibitors, we initially determined if MEK1/2 inhibitors can reduce cellular lipid accumulation in an ABCG1-dependent manner. RAW264.7 or peritoneal macrophages were transfected with scrambled siRNA or ABCG1 siRNA followed by treatment with U0126 and PD98059. Both ABCG1 mRNA and protein levels were substantially reduced by ABCG1 siRNA in RAW264.7 cells and peritoneal macrophages (PM ϕ , Fig. 3A). Oil Red O staining demonstrates that inhibition of ABCG1 expression by ABCG1 siRNA substantially increased RAW264.7 macrophage lipid accumulation (left control column, Fig. 3B) indicating the critical role of ABCG1 expression in prevention of macrophage/foam cell formation. MEK1/2 inhibitors reduced lipid accumulation in scrambled siRNA transfected cells while the reduction was attenuated in ABCG1 siRNA transfected cells (Figs. 3B, 3C). Similarly, in peritoneal macrophages, both PD98059 and U0126 inhibited foam cell formation

while the inhibition was attenuated when ABCG1 expression was inhibited by ABCG1 siRNA (Fig. 3D). Therefore, reduction of macrophage lipid accumulation by MEK1/2 inhibitors can be partially attributed by the activation of ABCG1 expression.

To determine the effect of MEK1/2 inhibitors on cholesterol efflux to HDL, [³H]-cholesterol radiolabeled peritoneal macrophages were treated with PD98059 and U0126 followed by incubation in medium containing HDL or not. As shown in Fig. 4A, the cholesterol efflux to HDL in control cells is dependent on HDL concentrations in the medium (The cholesterol efflux to “no HDL added medium” was considered as background, and subtracted from groups with “HDL added medium”. Thus, the net results after subtraction should be the cholesterol efflux to HDL). Furthermore, the cholesterol efflux to HDL was increased when cells received PD98059 or U0126 treatment suggesting that MEK1/2 inhibitors enhance another ABCG1 function. Besides the induction of cholesterol efflux to HDL, we and others have reported that MEK1/2 inhibitors also enhance macrophage cholesterol efflux to apoA1 due to the fact that MEK1/2 inhibitors are able to activate macrophage ABCA1 expression^{18, 21}.

To determine if MEK1/2 inhibitor can influence RCT *in vivo*, C57BL/6 wild type mice were fed normal chow or chow containing U0126 (3 mg/100 g food) for two weeks followed by i.p. injection of [³H]-cholesterol labeled peritoneal macrophages and determination of excreted radioactivity in the feces. Fig. 4B (left half) shows that [³H]-tracer in fecal samples was increased by U0126 administration over time. The radioactivity in serum and liver was also increased (Fig. 4B, right half) suggesting that U0126 treatment enhances RCT *in vivo*. Furthermore, after RCT study, we collected

mouse peritoneal macrophages and determined that U0126 treatment increased ABCG1 mRNA expression in those cells (left half, Fig. 4C) indicating that the increased RCT by U0126 might be contributed, at least in part by increased macrophage ABCG1 expression.

To determine if induction of macrophage ABCG1 expression by MEK1/2 inhibitors can inhibit atherosclerotic lesions in the aortic root, apoE^{-/-} mice were fed an atherogenic diet or the atherogenic diet containing U0126 (3 mg/100 g food) for 16 weeks. As shown in Fig. 4D, the sinus lesions in aortic root were substantially reduced by U0126 treatment. Associated with reduction of sinus lesions, the immunofluorescent staining demonstrates that U0126 significantly increased ABCG1 expression in lesion areas. Furthermore, the increased ABCG1 expression co-localized well with a macrophage marker, MOMA2, suggesting the reduction of lesions should be associated with the induction of macrophage ABCG1 expression in the arterial wall (Fig. 4E).

MEK1/2 inhibitors and RXR ligand coordinately induce macrophage ABCG1 expression

MEK1/2 inhibitors can synergize LXR ligand induced macrophage ABCA1 expression¹⁸. To determine if MEK1/2 inhibitors can function in a similar way on ABCG1 expression, we treated macrophages with MEK1/2 inhibitors at different concentrations or plus T0901317 (10 nM). Reciprocally, we treated cells with T0901317 at different concentrations or plus MEK1/2 inhibitors (40 μ M PD98059 and 1

μM U0126, respectively). The results in Figs. 5A and 5B (top panels) show that co-treatment of T0901317 with PD98059 or U0126 at different concentrations increased ABCG1 expression in an additive manner. Similar results were obtained with co-treatment of T0901317 at different concentrations and PD98059 or U0126 (Figs. 5A, 5B, bottom panels). Therefore, we did not observe the synergistic effect on ABCG1 expression by co-treatment with MEK1/2 inhibitors and LXR ligand.

LXR and RXR coordinately activate transcription of LXR target genes while RXR ligand alone is also able to activate LXR target gene transcription²². Therefore, we determined that treatment of macrophages with 9-*cis*-retenoic acid (9-*cis*-RA), an RXR agonist, increased ABCG1 mRNA and protein expression (Fig. 5C). Interestingly, co-treatment of cells with MEK1/2 inhibitors and 9-*cis*-RA demonstrates a coordinated induction of ABCG1 expression (Fig. 5D) suggesting that MEK1/2 inhibitors can enhance LXR activity.

MEK1/2 inhibitors induce macrophage ABCG1 expression by activating SIRT1 expression and inactivating SULT2B1 and RIP140 expression

LXR activity can be regulated, either positively or negatively, by several pathways. The deacetylase sirtuin 1 (SIRT1) reduces LXR acetylation that leads to LXR activation²³. To investigate if the induction of ABCG1 expression by MEK1/2 inhibitors is related to SIRT1-mediated LXR activation, we initially assessed the effects of MEK1/2 inhibitors on SIRT1 expression and activation. Fig. 6A demonstrates that PD98059 and U0126 increased SIRT1 mRNA expression. Cleavage of SIRT1 by

caspace-1 can generate a ~80 kDa species of SIRT1 which substantially down-regulates SIRT1 activity²⁴. In this study, we determined that both PD98059 and U0126 increased the full-length SIRT1 protein while reduced the 80 kDa SIRT1 species (Fig. 6B). Thus, MEK1/2 inhibitors can reduce SIRT1 cleavage indicating another mechanism activating SIRT1. In addition, administration of U0126 to mice activated macrophage SIRT1 expression *in vivo* (right half, Fig. 4C).

Correspondingly, treatment of macrophages with a SIRT1 activator (resveratrol) activated SIRT1 expression which was accompanied by increased ABCG1 mRNA expression (left half, Fig. 6C). In contrast, the SIRT1 inhibitor (nicotinamide) reduced both SIRT1 and ABCG1 mRNA expression (right half, Fig. 6C). Similarly, resveratrol increased while nicotinamide decreased ABCG1 protein expression, respectively (Fig. 6D). Specific inhibition of SIRT1 expression by SIRT1 siRNA also decreased ABCG1 mRNA levels (Fig. 6E) which further confirms that SIRT1 can regulate ABCG1 expression. Furthermore, nicotinamide abolished ABCG1 expression induced by MEK1/2 inhibitors (Fig. 6F). Therefore, induction of macrophage ABCG1 expression by MEK1/2 inhibitors is also associated with activation of SIRT1 expression/function.

Sulfotransferase family 2B member 1 (SULT2B1) catalyzes the transfer of sulfate group to oxysterols thereby inactivating oxysterols as LXR ligands^{25, 26}. We determined that both PD98059 and U0126 substantially inhibited SULT2B1 expression (Fig. 6G) which may block the suppression of SULT2B1 on LXR activity. The interaction between LXR and the co-repressor, receptor interacting protein 140 (RIP140), results in decreased LXR activity²⁷. Our study demonstrates that MEK1/2

inhibitors also greatly inhibited RIP140 mRNA expression (Fig. 6H) suggesting another pathway to activate LXR/ABCG1 expression. Taken together, the results in Fig. 6 suggest that activation of ABCG1 expression by MEK1/2 inhibitors might be completed by activating SIRT1 while inhibiting both SULT2B1 and RIP140 expression.

Discussion

In the animal models, overexpression and activation of ERK1/2 in atherosclerotic lesions of cholesterol-fed rabbits has been determined, while U0126 synergizes T0901317-inhibited atherosclerosis with elimination of LXR-induced deleterious effects in apoE^{-/-} mice^{17, 19}. These studies directly demonstrate the involvement of ERK1/2 in atherosclerosis, and the anti-atherogenic properties of MEK1/2 inhibitors. However, the underlying mechanisms for the anti-atherogenic properties of ERK1/2 inhibition have not been fully defined. We previously determined that MEK1/2 inhibitors alone can induce macrophage ABCA1 expression and ABCA1-mediated cholesterol efflux to apoA1¹⁸. In addition, MEK1/2 inhibitors synergize LXR-induced ABCA1 expression¹⁸. In this study, we found that MEK1/2 inhibitors induced macrophage ABCG1 expression (Figs. 1, 2A-2D) and ABCG1-mediated cholesterol efflux to HDL *in vitro* (Fig. 4A). At the cellular level, MEK1/2 inhibitors reduced macrophage/foam cell formation, partially in ABCG1-dependent manner (Fig. 3). *In vivo*, MEK1/2 inhibitor activated RCT and expression of ABCG1/SIRT1 while it inhibited the development of aortic sinus lesions which was associated with increased macrophage ABCG1 expression in lesion areas (Figs. 4B-4E). In addition to induction of ABCG1 expression through LXR activation, ERK1/2 inhibition is able to influence expression of other cholesterol trafficking molecules, such as ABCA1 and scavenger receptor type BI (SR-BI, the receptor for HDL to promote cholesterol efflux and RCT). This can occur in multiple cell type/tissues with involvement of different signaling pathways, such as LXR and peroxisome proliferator-activated receptor α and γ

(PPAR α/γ)^{18, 21, 28-30}. All of the above actions by ERK1/2 inhibition, particularly in combination, contribute to MEK1/2 inhibitor mediated enhancement of RCT, and inhibition of macrophage/foam cell formation and atherosclerosis.

Several other studies have demonstrated the anti-atherogenic functions of reduced ERK1/2 activity. C-reactive protein (CRP) may play a direct role in atherogenesis since it inhibits macrophage ABCA1/ABCG1 expression and cholesterol efflux³¹. The proinflammatory interleukin (IL)-17 induces CRP expression in hepatocytes, in part, by activating ERK1/2. Therefore, such induction can be attenuated by PD98059³². The induction of CRP expression in vascular endothelial cells by leptin can also be attenuated by PD98059³³. *In vivo*, the activation of peroxisome proliferator-activated receptor δ by ligand (GW0742) can attenuate angiotensin II-accelerated atherosclerosis in LDLR^{-/-} mice which is related to reduction of ERK1/2 activity³⁴. In contrast, elevated S-adenosyl-homocysteine induces vascular smooth muscle cell proliferation and migration through an oxidative stress-dependent activation of ERK1/2 pathway to promote atherogenesis³⁵.

Expression of macrophage ABCA1 and ABCG1 can be activated by both LXR ligand and MEK1/2 inhibitors. Compared to induction of ABCA1 expression which does not occur at the transcriptional level and it is unrelated to LXR activity¹⁸, MEK1/2 inhibitors activated ABCG1 promoter activity in an LXR dependent manner (Fig. 2E). In addition, MEK1/2 inhibitors synergized LXR-induced ABCA1¹⁸ but not ABCG1 expression (Figs. 5A, 5B). RXR ligand alone induced ABCG1 expression (Fig. 5C). It also coordinated with MEK1/2 inhibitor-induced ABCG1 expression (Fig. 5D) which

implies that MEK1/2 inhibitors may function as putative LXR ligands regarding the regulation of ABCG1 expression. However, our previous study demonstrated several differences between T0901317 and MEK1/2 inhibitors³⁶. By completing a “molecular docking study”, we determined that MEK1/2 inhibitors had much lower affinity for LXR protein than T0901317. Taken together, these results suggest that MEK1/2 inhibitors activate LXR targeted gene expression by mechanisms other than LXR ligands.

SIRT1 deacetylates LXR to activate LXR while lack of SIRT1 expression reduces LXR target gene expression including ABCA1 and ABCG1²³. Interestingly, SIRT1 activation inhibits atherosclerosis in apoE^{-/-} mice by reducing hepatic PCSK9 secretion and enhancing LDLR expression³⁷. Specific inhibition of SIRT1 in vascular smooth muscle cells enhances lesion development by increasing DNA damage³⁸. Therefore, these studies indicate that SIRT1 may inhibit atherosclerosis by different mechanisms. In our study, we determined that MEK1/2 inhibitors increased SIRT1 expression while reducing the cleavage of SIRT1 which can further enhance SIRT1 activity (Figs. 6A, 6B). Moreover, inhibition of SIRT1 antagonized MEK1/2 inhibitor-induced SIRT1 and ABCG1 expression (Fig. 6F). In contrast, it has been reported that inhibition of SIRT1 by nicotinamide/sirtinol or siRNA reduces phosphorylation of ERK1/2 which results in protection of neurons against oxidative damage³⁹ while SIRT1 overexpression activates ERK1/2 in the striatum⁴⁰. These studies demonstrate a direct interaction between SIRT1 and ERK1/2. In our study, we believe that SIRT1 activation by MEK1/2 inhibitors suggests a feedback inhibitory mechanism by which the effect of SIRT1 on ERK1/2 phosphorylation can be kept

under control since SIRT1 activation by MEK1/2 inhibitors can, in turn, neutralize and prevent irreversible inhibition of ERK1/2 by the small molecular MEK1/2 inhibitors. In addition, we determined that MEK1/2 inhibitors inhibited expression of SULT2B1 and RIP140 (Figs. 6G, 6H), two molecules inactivating LXR. Our previous study demonstrates that MEK1/2 inhibitors increase LXR expression/nuclear translocation³⁶ which might be a mechanism by which MEK1/2 inhibitors activate LXR. Taken together, the above findings indicate that multiple mechanisms are involved in MEK1/2 inhibitor-activated macrophage ABCG1 expression/functions (Fig. S1).

Synthetic ligands, such as T0901317 and GW3965, can inhibit atherosclerosis⁴¹,⁴². However, the lipogenic effects limit application of LXR ligands for atherosclerosis treatment⁴³. We previously reported that the combination of T0901317 and U0126 can reduce atherosclerosis in a synergistic manner while blocking LXR-induced lipogenesis¹⁹. Our current study advances the field by showing a role for MEK1/2 inhibitors in activation of macrophage ABCG1 expression/functions. Such findings demonstrate an additional anti-atherogenic mechanism of MEK1/2 inhibitors which can further support the concept that the combination of MEK1/2 inhibitor and LXR ligand might be a novel therapeutic approach for atherosclerosis treatment.

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Conflict of interest

None declared

References

1. Fuster V, Voute J, Hunn M, Smith SC, Jr. Low priority of cardiovascular and chronic diseases on the global health agenda: A cause for concern. *Circulation*. 2007;**116**:1966-1970
2. Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature*. 1993;**362**:801-809
3. de Villiers WJ, Smart EJ. Macrophage scavenger receptors and foam cell formation. *J Leukoc Biol*. 1999;**66**:740-746
4. Simons K, Ikonen E. How cells handle cholesterol. *Science*. 2000;**290**:1721-1726
5. Wang N, Lan D, Chen W, Matsuura F, Tall AR. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc Natl Acad Sci U S A*. 2004;**101**:9774-9779
6. Wang N, Ranalletta M, Matsuura F, Peng F, Tall AR. LXR-induced redistribution of ABCG1 to plasma membrane in macrophages enhances cholesterol mass efflux to HDL. *Arterioscler Thromb Vasc Biol*. 2006;**26**:1310-1316
7. Out R, Hoekstra M, Hildebrand RB, Kruit JK, Meurs I, Li Z, Kuipers F, Van Berkel TJ, Van Eck M. Macrophage ABCG1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. 2006;**26**:2295-2300
8. Yvan-Charvet L, Ranalletta M, Wang N, Han S, Terasaka N, Li R, Welch C, Tall

- AR. Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. *J Clin Invest.* 2007;**117**:3900-3908
9. Out R, Hoekstra M, Habets K, Meurs I, de Waard V, Hildebrand RB, Wang Y, Chimini G, Kuiper J, Van Berkel TJ, Van Eck M. Combined deletion of macrophage ABCA1 and ABCG1 leads to massive lipid accumulation in tissue macrophages and distinct atherosclerosis at relatively low plasma cholesterol levels. *Arterioscler Thromb Vasc Biol.* 2008;**28**:258-264
 10. Larrede S, Quinn CM, Jessup W, Frisdal E, Olivier M, Hsieh V, Kim MJ, Van Eck M, Couvert P, Carrie A, Giral P, Chapman MJ, Guerin M, Le Goff W. Stimulation of cholesterol efflux by LXR agonists in cholesterol-loaded human macrophages is ABCA1-dependent but ABCG1-independent. *Arterioscler Thromb Vasc Biol.* 2009;**29**:1930-1936
 11. Meurs I, Lammers B, Zhao Y, Out R, Hildebrand RB, Hoekstra M, Van Berkel TJ, Van Eck M. The effect of ABCG1 deficiency on atherosclerotic lesion development in LDL receptor knockout mice depends on the stage of atherogenesis. *Atherosclerosis.* 2012;**221**:41-47
 12. Sabol SL, Brewer HB, Jr., Santamarina-Fojo S. The human ABCG1 gene: Identification of LXR response elements that modulate expression in macrophages and liver. *J Lipid Res.* 2005;**46**:2151-2167
 13. Janowski BA, Grogan MJ, Jones SA, Wisely GB, Kliewer SA, Corey EJ, Mangelsdorf DJ. Structural requirements of ligands for the oxysterol liver X

- receptors LXRalpha and LXRbeta. *Proc Natl Acad Sci U S A*. 1999;**96**:266-271
14. Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, Lustig KD, Shan B. Role of LXRs in control of lipogenesis. *Genes Dev*. 2000;**14**:2831-2838
 15. Zheng CF, Guan KL. Dephosphorylation and inactivation of the mitogen-activated protein kinase by a mitogen-induced Thr/Tyr protein phosphatase. *J Biol Chem*. 1993;**268**:16116-16119
 16. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endoc Rev*. 2001;**22**:153-183
 17. Hu Y, Dietrich H, Metzler B, Wick G, Xu Q. Hyperexpression and activation of extracellular signal-regulated kinases (ERK1/2) in atherosclerotic lesions of cholesterol-fed rabbits. *Arterioscler Thromb Vasc Biol*. 2000;**20**:18-26
 18. Zhou X, Yin Z, Guo X, Hajjar DP, Han J. Inhibition of ERK1/2 and activation of liver X receptor synergistically induce macrophage ABCA1 expression and cholesterol efflux. *J Biol Chem*. 2010;**285**:6316-6326
 19. Chen Y, Duan Y, Yang X, Sun L, Liu M, Wang Q, Ma X, Zhang W, Li X, Hu W, Miao RQ, Xiang R, Hajjar DP, Han J. Inhibition of ERK1/2 and activation of LXR synergistically reduce atherosclerotic lesions in apoe-deficient mice. *Arterioscler Thromb Vasc Biol*. 2015;**35**:948-959
 20. Han J, Hajjar DP, Febbraio M, Nicholson AC. Native and modified low density lipoproteins increase the functional expression of the macrophage class B

- scavenger receptor, CD36. *J Biol Chem.* 1997;**272**:21654-21659
21. Mulay V, Wood P, Manetsch M, Darabi M, Cairns R, Hoque M, Chan KC, Reverter M, Alvarez-Guaita A, Rye KA, Rentero C, Heeren J, Enrich C, Grewal T. Inhibition of mitogen-activated protein kinase ERK1/2 promotes protein degradation of atp binding cassette transporters A1 and G1 in cho and Huh7 cells. *PloS One.* 2013;**8**:e62667
 22. Murthy S, Born E, Mathur SN, Field FJ. LXR/RXR activation enhances basolateral efflux of cholesterol in Caco-2 cells. *J Lipid Res.* 2002;**43**:1054-1064
 23. Li X, Zhang S, Blander G, Tse JG, Krieger M, Guarente L. SIRT1 deacetylates and positively regulates the nuclear receptor LXR. *Mol Cell.* 2007;**28**:91-106
 24. Chalkiadaki A, Guarente L. High-fat diet triggers inflammation-induced cleavage of SIRT1 in adipose tissue to promote metabolic dysfunction. *Cell Met.* 2012;**16**:180-188
 25. Chen WL, Chen GX, Head DL, Mangelsdorf DJ, Russell DW. Enzymatic reduction of oxysterols impairs LXR signaling in cultured cells and the livers of mice. *Cell Met.* 2007;**5**:73-79
 26. Bensinger SJ, Bradley MN, Joseph SB, Zelcer N, Janssen EM, Hausner MA, Shih R, Parks JS, Edwards PA, Jamieson BD, Tontonoz P. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. *Cell.* 2008;**134**:97-111
 27. Jakobsson T, Osman W, Gustafsson JA, Zilliacus J, Warnmark A. Molecular

- basis for repression of liver X receptor-mediated gene transcription by receptor-interacting protein 140. *Biochem J.* 2007;**405**:31-39
28. Agassandian M, Miakotina OL, Andrews M, Mathur SN, Mallampalli RK. *Pseudomonas aeruginosa* and sPLA2 IB stimulate ABCA1-mediated phospholipid efflux via ERK-activation of PPARalpha-RXR. *Biochem J.* 2007;**403**:409-420
 29. Wood P, Mulay V, Darabi M, Chan KC, Heeren J, Pol A, Lambert G, Rye KA, Enrich C, Grewal T. Ras/mitogen-activated protein kinase (MAPK) signaling modulates protein stability and cell surface expression of scavenger receptor SR-BI. *J Biol Chem.* 2011;**286**:23077-23092
 30. Mogilenko DA, Shavva VS, Dizhe EB, Orlov SV, Perevozchikov AP. PPARgamma activates ABAC1 gene transcription but reduces the level of ABCA1 protein in HepG2 cells. *Biochem Biophys Res Commun.* 2010;**402**:477-482
 31. Wang X, Liao D, Bharadwaj U, Li M, Yao Q, Chen C. C-reactive protein inhibits cholesterol efflux from human macrophage-derived foam cells. *Arterioscler Thromb Vasc Biol.* 2008;**28**:519-526
 32. Patel DN, King CA, Bailey SR, Holt JW, Venkatachalam K, Agrawal A, Valente AJ, Chandrasekar B. Interleukin-17 stimulates C-reactive protein expression in hepatocytes and smooth muscle cells via p38 MAPK and ERK1/2-dependent NF-kappaB and c/EBPbeta activation. *J Biol Chem.* 2007;**282**:27229-27238
 33. Singh P, Hoffmann M, Wolk R, Shamsuzzaman AS, Somers VK. Leptin induces

- C-reactive protein expression in vascular endothelial cells. *Arterioscler Thromb Vasc Biol.* 2007;**27**:e302-307
34. Takata Y, Liu J, Yin F, Collins AR, Lyon CJ, Lee CH, Atkins AR, Downes M, Barish GD, Evans RM, Hsueh WA, Tangirala RK. PPARdelta-mediated antiinflammatory mechanisms inhibit angiotensin ii-accelerated atherosclerosis. *Proc Natl Acad Sci U S A.* 2008;**105**:4277-4282
 35. Luo X, Xiao Y, Song F, Yang Y, Xia M, Ling W. Increased plasma s-adenosyl-homocysteine levels induce the proliferation and migration of vsmcs through an oxidative stress-ERK1/2 pathway in apoE(-/-) mice. *Cardiovasc Res.* 2012;**95**:241-250
 36. Ma X, Wang Q, Liu Y, Chen Y, Zhang L, Jiang M, Li X, Xiang R, Miao RQ, Duan Y, Han J. Inhibition of tumor growth by U0126 is associated with induction of interferon-gamma production. *Int J Cancer.* 2015;**136**:771-783
 37. Miranda MX, van Tits LJ, Lohmann C, Arsiwala T, Winnik S, Tailleux A, Stein S, Gomes AP, Suri V, Ellis JL, Lutz TA, Hottiger MO, Sinclair DA, Auwerx J, Schoonjans K, Staels B, Luscher TF, Matter CM. The SIRT1 activator SRT3025 provides atheroprotection in apoE^{-/-} mice by reducing hepatic PCSK9 secretion and enhancing LDLR expression. *Eur Heart J.* 2015;**36**:51-59
 38. Gorenne I, Kumar S, Gray K, Figg N, Yu H, Mercer J, Bennett M. Vascular smooth muscle cell sirtuin 1 protects against DNA damage and inhibits atherosclerosis. *Circulation.* 2013;**127**:386-396
 39. Li Y, Xu W, McBurney MW, Longo VD. SIRT1 inhibition reduces

- IGF-I/IRS-2/RAS/ERK1/2 signaling and protects neurons. *Cell Met.* 2008;**8**:38-48
40. Wu D, Qiu Y, Gao X, Yuan XB, Zhai Q. Overexpression of SIRT1 in mouse forebrain impairs lipid/glucose metabolism and motor function. *PloS One.* 2011;**6**:e21759
41. Joseph SB, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, Tran J, Tippin TK, Wang X, Lusis AJ, Hsueh WA, Law RE, Collins JL, Willson TM, Tontonoz P. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci U S A.* 2002;**99**:7604-7609
42. Terasaka N, Hiroshima A, Koieyama T, Ubukata N, Morikawa Y, Nakai D, Inaba T. T-0901317, a synthetic liver X receptor ligand, inhibits development of atherosclerosis in LDL receptor-deficient mice. *FEBS Letters.* 2003;**536**:6-11
43. Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev.* 2000;**14**:2819-2830

Figure legends

Fig. 1. MEK1/2 inhibitors induce macrophage ABCG1 protein expression

A, B: RAW264.7 macrophages were treated with PD98059, U0126 and T0901317 at the indicated concentrations for 16 h, or with PD98059 (10 μ M) and U0126 (1 μ M) for the indicated times; C, D: peritoneal macrophages were treated with PD98059 (PD), U0126 (U0) and T0 at the indicated concentrations for 16 h, or with PD98059 (10 μ M) and U0126 (1 μ M) for the indicated times; E: THP-1/PMA macrophages were treated with PD98059, U0126 and T0 at the indicated concentrations for 16 h. Expression of ABCG1 protein was determined by immunofluorescent staining with intact cells (C, low panel; bars, 100 μ m) or Western blot with total cellular protein extract. MFI indicates the mean of fluorescent intensity of the images (n=6).

Fig. 2. MEK1/2 inhibitors activate macrophage ABCG1 transcription

A, B: RAW264.7 macrophages were treated with PD98059 and U0126 at the indicated concentrations for 14 h or with PD98059 (40 μ M) and U0126 (1 μ M) for the indicated times. Expression of ABCG1 mRNA was determined by Northern blot; C, D: peritoneal macrophages were treated with PD98059 and U0126 at the indicated concentrations for 16 h, or with PD98059 (10 μ M) and U0126 (1 μ M) for the indicated times. Expression of ABCG1 mRNA was determined by real time RT-PCR. *: P<0.05, **: P<0.01 vs. control (n=3); E: 293T cells were transfected with the natural ABCG1 promoter (*pABCG1*) or the ABCG1 promoter with DR4 (LXRE) deletion (*pABCG1-DR4-del*) plus LXR β expression vector and *Renilla* (as an internal control)

for 4 h followed by the indicated treatment overnight. Activity of firefly and Renilla luciferases in cellular lysate was determined using the Dual-Luciferase Reporter Assay system. *: $P < 0.05$ vs. promoter alone ($n=3$); #: $P < 0.05$ vs. *pABCG1*; F: RAW264.7 macrophages received the indicated treatment for 16 h. Cells were then used to isolate chromatin by sonication followed by immunoprecipitation with normal IgG or anti-LXR antibody as indicated. The PCR was conducted with the primers for the LXRE in the ABCG1 promoter. The density ratio of PCR product of LXRE for the immunoprecipitation (IP) to input in control group was defined as 100% for the statistical analysis (right panel), *: $P < 0.05$ vs. control ($n=3$).

Fig. 3. MEK1/2 inhibitors reduce foam cell formation by activating ABCG1 expression

A: RAW264.7 cells or peritoneal macrophages were transfected with control/scrambled siRNA (si-Ctrl) or the siRNA against ABCG1 (si-ABCG1) for 24 h. The transfected cells were then switched into complete medium for another 48 h followed by determination of ABCG1 mRNA and protein expression with real time RT-PCR and Western blot, respectively. *: $P < 0.05$ vs. si-Ctrl ($n=3$); B: si-Control and si-ABCG1 transfected RAW264.7 cells were treated with U0126 and PD98059 at the indicated concentrations for 8 h, respectively. The cells were incubated with 25 $\mu\text{g/ml}$ AcLDL for 8 h. After AcLDL was removed, cells were incubated with 15 $\mu\text{g/ml}$ HDL for another 8 h followed by Oil Red O staining; C: the accumulated Oil Red O dye within macrophages shown in Figure 3B was extracted and quantified. *: $P < 0.05$, **: $P < 0.01$ vs. control (un-treated) in the corresponding group ($n=3$); D: peritoneal macrophages

isolated from apoE^{-/-} mice were transfected with si-Control and si-ABCG1 and then treated with U0126 (2 μ M) and PD98059 (20 μ M) for 8 h. Cellular lipid content was determined by Oil Red O staining and quantitation of the accumulated Oil Red O dye using the same procedure to RAW264.7 cells as above described. *: P<0.05, **: P<0.01 vs. control (un-treated) in the corresponding group (n=3).

Fig. 4. Regulation of macrophage cholesterol efflux, RCT, and sinus lesions and ABCG1 expression in aortic root by MEK1/2 inhibitors

A: peritoneal macrophages were isolated from C57BL/6 wild type mice and pre-labeled with [³H]-cholesterol. After treatment with PD98059 (PD) and U0126 (U0) at the indicated concentrations overnight, cells were incubated with medium containing HDL at the indicated concentrations for 5 h followed by determination of radioactivity in the medium and cellular protein in the corresponding sample which was used to normalize cholesterol efflux to HDL (CPM/ μ g cellular protein). The cholesterol efflux in group incubated in the medium no HDL added was considered as background, and it was subtracted from groups incubated in the medium with HDL added. *: P<0.05 vs. control in the corresponding group (n=3); B, C: C57BL/6 wild type mice were randomly divided into two groups (5/group) and fed normal chow or normal chow containing U0126 (3 mg/100 g food) for 2 weeks. Each mouse was then i.p. injected with pre-radiolabeled macrophages followed by *in vivo* RCT determination (B); mouse peritoneal macrophages were collected at the end of RCT, and used to determine expression of ABCG1 and SIRT1 mRNA by real time RT-PCR. *: P<0.05 vs.

control (n=5); D: apoE^{-/-} mice (15/group) were fed an atherogenic diet or an atherogenic diet containing U0126 (3 mg/100 g food) for 16 weeks. Aortic root cross sections were used to determine sinus lesions by Oil Red O staining. *: P<0.05 vs. control (n=15); E: expression of ABCG1 and MOMA2 in aortic root cross section was determined by immunofluorescent staining. The white arrowheads indicate the co-localization of ABCG1 and MOMA2. Bars, 100 μm.

Fig. 5. MEK1/2 inhibitors and RXR ligand coordinately induce macrophage ABCG1 expression

A, B: RAW264.7 macrophages were treated with T0901317 (T0), PD98059 (PD), U0126 (U0) or their combination as indicated overnight. ABCG1 protein expression was determined by Western blot; C: RAW264.7 macrophages were treated with 9-*cis*-retinoic acid (9-*cis*-RA) at the indicated concentrations overnight; D: RAW264.7 macrophages received 9-*cis*-RA, or 9-*cis*-RA plus PD98059, U0126 or T0901713 at the indicated concentrations overnight. Expression of ABCG1 mRNA and protein in C and D were determined by Northern blot and Western blot, respectively.

Fig. 6. Induction of ABCG1 protein by MEK1/2 inhibitors is related to activation of SIRT1, and inhibition of SULT2B1 and RIP140 expression

A, B: RAW264.7 macrophages were treated with PD98059 and U0126 at the indicated concentrations overnight. Expression of SIRT1 mRNA and protein (both full length and the 80 kDa species) were determined by real time RT-PCR and Western

blot, respectively. *: $P < 0.05$ vs. control (n=3); C, D: RAW264.7 cells were treated with resveratrol and nicotinamide at the indicated concentrations overnight. Expression of SIRT1 mRNA and ABCG1 mRNA or protein were determined by real time RT-PCR and Western blot, respectively. *, §: $P < 0.05$ vs. control in the corresponding group (n=3); E: RAW264.7 cells were transfected with scrambled siRNA (Ctrl) and siRNA against SIRT1 (SIRT1) followed by determination of SIRT1 and ABCG1 mRNA expression by real time RT-PCR. *, §: $P < 0.05$ vs. Ctrl in the corresponding group (n=3); F: RAW264.7 cells were treated with PD98059 and U0126 or plus nicotinamide at the indicated concentrations overnight. Expression of SIRT1 and ABCG1 mRNA was determined by real time RT-PCR. *, §: $P < 0.05$ vs. control in the corresponding group (n=3); G: RAW264.7 cells were treated with PD98059 and U0126 at the indicated concentrations overnight. Expression of SULT2B1 was determined by real time RT-PCR. *: $P < 0.05$ vs. control (n=3); H: RAW264.7 cells were treated with U0126 and PD98059 at the indicated concentrations overnight, respectively. Expression of RIP140 was determined by real time RT-PCR. *: $P < 0.05$ vs. control (n=3).

Table 1. Sequences of primers for real time RT-PCR

Gene	Sense	Anti-sense
<i>ABCG1</i>	5'-CCTACTCTGTACCCGAGGGG-3'	5'-CTTCCCAGCTCCAGAAGGAC-3'
<i>GAPDH</i>	5'-ACCCAGAAGACTGTGGATGG-3'	5'-ACACATTGGGGGTAGGAACA-3'
<i>RIP140</i>	5'-CAACAGCCTTCTCAGCTTCC-3'	5'-TCATCTTTCGTTGCTCACCA-3'
<i>SIRT1</i>	5'-GACGGTATCTATGCTCGCCT-3'	5'-ACACAGAGACGGCTGGAAC-3'
<i>SULT2B1</i>	5'-CGGGACGACGACATCTTCA-3'	5'-TAAGCAGACGATCTCAATCATCCA-3'

Zhang *et al*, Figure 2

Zhang *et al*, Figure 3

Zhang et al, Figure 4

Zhang *et al*, Figure 5

Zhang *et al*, Figure 6