Published in final edited form as:

Biochemistry. 2015 November 24; 54(46): 6931–6941. doi:10.1021/acs.biochem.5b00894.

LXR Agonism Upregulates the Macrophage ABCA1/Syntrophin Protein Complex That Can Bind ApoA-I and Stabilized ABCA1 Protein, but Complex Loss Does Not Inhibit Lipid Efflux

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Abstract

Macrophage ABCA1 effluxes lipid and has anti-inflammatory activity. The syntrophins, which are cytoplasmic PDZ protein scaffolding factors, can bind ABCA1 and modulate its activity. However, many or “much” of the data assessing the function of the ABCA1–syntrophin interaction are based on overexpression in nonmacrophage cells. To assess endogenous complex function in macrophages, we derived immortalized macrophages from *Abca1*^{+/+} and *Abca1*^{-/-} mice and show their phenotype recapitulates primary macrophages. *Abca1*^{+/+} lines express the CD11B and F4/80 macrophage markers and markedly upregulate cholesterol efflux in response to LXR nuclear hormone agonists. In contrast, immortalized *Abca1*^{-/-} macrophages show no efflux to apoA-I. In response to LPS, *Abca1*^{-/-} macrophages display pro-inflammatory changes, including an increased level of expression of cell surface CD14, and 11–26-fold higher levels of IL-6 and IL-12 mRNA. Given recapitulation of phenotype, we show with these lines that the ABCA1–syntrophin protein complex is upregulated by LXR agonists and can bind apoA-I. Moreover, in immortalized macrophages, combined $\alpha 1/\beta 2$ -syntrophin loss modulated ABCA1 cell surface levels and induced pro-inflammatory gene expression. However, loss of all three syntrophin isoforms known to bind

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00894.

Figure S1 (PDF)

Figure S2 (PDF)

Notes

The authors declare no competing financial interest.

ABCA1 did not impair lipid efflux in immortalized or primary macrophages. Thus, the ABCA1–syntrophin protein complex is not essential for ABCA1 macrophage lipid efflux but does directly interact with apoA-I and can modulate the pool of cell surface ABCA1 stabilized by apoA-I.

Graphical abstract

Macrophages function as professional phagocytes engulfing both cellular and acellular debris.¹ Consequently, they are exposed to large and varying influxes of cholesterol. Because, in excess, engulfed free cholesterol is cytotoxic, it is esterified by macrophages and stored in lipid droplets, inclusions that give these cells a foamy appearance at the histologic level after staining with dyes such as Oil Red O. Importantly, the presence of macrophage foam cells in the subendothelial space of coronary arteries is one of the earliest hallmarks of atherosclerotic vascular disease. These macrophage foam cells are derived from circulating monocytes that extravasate in response to local inflammatory signals and subsequently become engorged with cholesterol derived from the uptake of modified LDL particles.² This binding and uptake of modified LDL by innate pattern recognition molecules such as CD-36, in concert with toll-like receptors, engages additional inflammatory signaling pathways, and this leads to the further release of cytokines by these subintimal macrophage foam cells.³ Thus, chronic cholesterol uptake and cytokine secretion by macrophages is an early and key process that drives atherosclerotic progression.

To prevent buildup of excess cholesterol, macrophages have evolved transporters to export this lipid by a mechanism termed cholesterol efflux. The ABCA1 transporter plays a rate-limiting role in this process, and its activity is critical for the biogenesis of HDL as evidenced by Tangier disease patients who carry loss of function ABCA1 mutations and have little or no circulating HDL.⁴ In these individuals, cholesterol-engorged macrophages accumulate in their peripheral tissues, and these individuals can suffer premature cardiovascular disease. Likewise, mice lacking ABCA1 also have little circulating HDL, and when bone marrow from these animals is transplanted into hyperlipidemic mice, this exacerbates atherosclerosis.^{5–9} Although the physiologic importance of ABCA1 is clear, the mechanism by which this large multitransmembrane protein stimulates cholesterol efflux and reduces inflammatory signaling is less so.

As part of the efflux mechanism, we and others have shown that ABCA1 binds apoA-I, the major apolipoprotein of HDL, to efflux cholesterol and phospholipid.^{10–14} This interaction between ABCA1 and apoA-I is driven by the amphipathic helices in apoA-I, and this is a promiscuous interaction in that ABCA1 can interact with a number of other proteins containing amphipathic helices, including apoA-II, apoE, and apoC-III.^{11,15,16} Moreover, ABCA1 can bind additional proteins that contain one to multiple PDZ domains because the

transporter contains a C-terminal cytoplasmic domain that has been conserved throughout evolution as a PDZ binding motif.^{17–20} PDZ proteins contain multiple protein–protein interaction domains; hence, they can scaffold membrane proteins such as ABCA1 into supramolecular assemblies. Using a proteomic approach, we previously found three syntrophin isoforms ($\alpha 1$, $\beta 1$, and $\beta 2$) copurified with ABCA1 by binding the C-terminal PDZ motif.²¹ When co-expressed with ABCA1 in HEK293 cells, the $\alpha 1$ - and $\beta 1$ -syntrophins stabilize newly synthesized ABCA1 protein and increase efflux activity.^{19,21} However, the role that endogenous syntrophins may play in the macrophage efflux process remains poorly described.

To address this question here, we have isolated primary bone marrow-derived macrophages from both *Abca1*^{−/−} and *Syntrophin* ^{$\alpha 1$ −/−/ $\beta 2$ −/−} mice and derived immortalized lines from these cells using a J2 retrovirus carrying the v-raf and v-myc oncogenes.²² We show that wild-type immortalized cells maintain the macrophage phenotype and continue to express ABCA1 in a LXR nuclear hormone-dependent manner. Immortalized *Abca1*^{−/−} macrophages lack apoA-I-dependent efflux and exhibited a partial defect in HDL-dependent efflux. They also display a pro-inflammatory response to bacterial lipopolysaccharide (LPS) that was associated with higher levels of cell surface CD-14, an increased level of expression of cytokines, and a higher level of foam cell formation after treatment with acetylated LDL. Given the cells phenocopy the behavior of primary *Abca1*^{−/−} macrophages, they and the *Syntrophin* ^{$\alpha 1$ −/−/ $\beta 2$ −/−} cells were used to study the function of the ABCA1–syntrophin complex. It was found that the complex was upregulated by treating cells with apoA-I or with LXR agonists, and that the ABCA1–syntrophin complex can form a tripartite interaction with apoA-I. Deletion of the $\alpha 1$ - and $\beta 2$ -syntrophins caused a partial weakening or “defect” in the ability of ABCA1 to interact with apoA-I and reduced ABCA1 protein stability and cell surface levels. However, these changes did not have a significant impact on lipid efflux activity to apoA-I, even when the expression of the third $\beta 1$ isoform was suppressed using lentivirus-delivered siRNAs in primary or immortalized macrophages.

EXPERIMENTAL PROCEDURES

Abca1^{−/−} and *Syntrophin* ^{$\alpha 1$ −/−/ $\beta 2$ −/−} Mice

Mice hetero zygous for a null allele at the *Abca1* locus on a DBA/1 × C57BL/6J hybrid background were obtained from Jackson Laboratories and were intercrossed to produce homozygous *Abca1*^{−/−} mice and littermate wild-type controls for these studies. *Syntrophin* ^{$\alpha 1$ −/−/ $\beta 2$ −/−} (*Syn* ^{$\alpha 1$ −/−/ $\beta 2$ −/−}) mice carrying null mutations at both the $\alpha 1$ - and $\beta 2$ -syntrophin loci have been back bred 10 generations into the C57BL/6J background and have been previously described.²³ All animal procedures were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and were conducted in accordance with the U.S. Department of Agriculture Animal Welfare Act and the PHS Policy for the Humane Care and Use of Laboratory Animals.

Immortalization of Bone Marrow-Derived Macrophages

Immortalized macrophage cell lines were derived by transducing bone marrow-derived myeloid precursors with a J2 recombinant retrovirus carrying the v-myc and v-raf oncogenes

as previously described.²² In brief, primary born marrow cultures were incubated in L929 mouse fibroblast-conditioned medium for 4 days to first induce macrophage differentiation. Subsequently, cells were infected with concentrated J2 virus and selected for growth in the absence of L929-conditioned medium. Clonal lines were established by limiting dilution from the initial mixed cultures, and their macrophage phenotype was verified by flow cytometry for CD11b and F4/80 antigen cell surface expression levels. Because the genetic background varied between the *Abca1*^{-/-} and *Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-} mouse strains, two control lines were established from the respective littermate wild-type animals. In the figures, these lines are denoted as *Abca1*^{+/+} or *Syn* ^{$\alpha 1$ +/+ $\beta 2$ +/+}. The results presented are representative of two or more distinct clones.

$\beta 1$ -Syntrophin mRNA Suppression

Primary screening of OpenBiosystems lentiviral constructs carrying small hairpin (sh) RNAs targeting murine $\beta 1$ -syntrophin mRNA showed clone RMM3981-98072652 had the greatest efficacy in reducing the level of expression of the $\beta 1$ -syntrophin protein as assessed by transient transfection of a mouse $\beta 1$ -syntrophin cDNA with the lentiviral clones. Particles generated by packaging this construct in 293 cells were used to infect primary mouse bone marrow cultures derived from the *Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-} mice, and cells expressing the shRNA targeting $\beta 1$ -syntrophin were obtained by selection with puromycin (4 μ g/mL, 48 h postinfection for 72 h). In parallel, *Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-} cells expressing a shRNA targeting green fluorescent protein (GFP) were also generated by lentiviral infection and puromycin treatment to control for off-target effects of the shRNAs and for the infection and selection process.

Flow Cytometry

Cells lifted from plates by gentle pipetting were washed twice with PBS containing 2% FBS and 0.1% NaN₃, and their Fc γ receptor was blocked by incubation for 15 min with 0.5 μ g/mL purified anti-mouse CD16/CD32 antibody (BD Biosciences). Cells were then incubated at 4 °C for 30 min with either 40 ng/mL PE-conjugated anti-mouse IgA to assess background binding or with a FITC-conjugated anti-mouse F4/80 antibody (eBio-science), a PE-conjugated anti-mouse CD11b antibody, a FITC-conjugated anti-mouse CD14 antibody (BD Biosciences), or an anti-ABCA1 rat monoclonal antibody (Affinity BioReagents). To visualize cell surface ABCA1, cells were additionally incubated with 40 ng/mL FITC-conjugated anti-rat IgG for 30 min. After the cells had been washed with 2% FBS and 0.1% NaN₃/PBS, cell fluorescence was determined using a FACSCalibur flow cytometer and analyzed with FlowJo software (BD Biosciences).

Lipid Efflux

Cholesterol efflux assays were conducted as previously described.¹⁸ In brief, primary and immortalized macrophages were cultured in conditioned (15% L929-conditioned medium/10% FBS/75% DMEM) or standard medium (10% FBS/90% DMEM), respectively. Twenty-four hours later, the cells were radiolabeled with 1 μ Ci/mL [³H]cholesterol in the presence of 10 μ g/mL cholesterol for 24 h, and unincorporated radiolabel and cholesterol were removed by a 2 h incubation in efflux medium (2 mg/mL fatty acid free BSA/DMEM) and two washes of 37 °C, 1 $\times$ PBS. Cells were further incubated in efflux medium for 20 h in

the presence of delipidated apoA-I or HDL (10 $\mu\text{g}/\text{mL}$), and either vehicle (DMSO) or 1 μM synthetic LXR agonist TO-901317 (Sigma). Medium was collected from the cells and cleared by a 2500g spin for 10 min; the cells were dissolved in 0.1 N NaOH, and the percent cholesterol efflux [medium counts per minute/(medium counts per minute + cell-associated counts per minute) $\times$ 100] was calculated by scintillation counting. Likewise, to measure the efflux of choline-containing phospholipids, cells were incubated with 1 $\mu\text{Ci}/\text{mL}$ [^3H -*methy*]choline, washed, and treated with the efflux acceptors and LXR agonists as described above, and media and cell lipids were extracted into a hexane/2-propanol mixture [3:2 (v:v)], dried, and subjected to scintillation counting. All assays were performed in triplicate and are representative of two or more independent experiments.

Oil Red O Staining and Total Cholesterol

To assess foam cell formation, cells cultured on glass coverslips for 2 days were treated with acetylated LDL (15 $\mu\text{g}/\text{mL}$) alone or with LPS (1 ng/mL) for 48 h, washed with PBS, fixed in 4% paraformaldehyde for 30 min, and stained with Oil Red O for 6 h (saturated solution in 60% 2-propanol). After being washed, slides were mounted and imaged by light microscopy. In parallel, to measure total cholesterol accumulation, cells (12 well plates, 250000 cells/well) were treated as described above on day 2, lysates were prepared, and total cholesterol was quantified using the Amplex Red Cholesterol Assay Kit (Life Technoloiges).

Cell Surface and Protein–Protein Interaction Assays

To assess the amount of apoA-I bound to ABCA1 and whether the ABCA1–syntrophin complex can interact with apoA-I, chemical cross-linking assays were conducted as previously described.^{11,12} In brief, lipid poor apoA-I (BioDesign) was radiolabeled with ^{125}I to a specific activity of 1000 cpm/ng using Iodo-Beads according to the manufacturer's instructions (Pierce). Unincorporated radionucleotides were eliminated by gel filtration, and the efficiency of the separation (99%) was determined by trichloroacetic acid precipitation. Cells were incubated for 1 h at 37 °C with [^{125}I]apoA-I (1 $\mu\text{g}/\text{mL}$ in 2 mg/mL fatty acid free BSA/DMEM), washed twice with warm 1 $\times$ PBS, and then chilled on ice and exposed to the thio-reducible DSP chemical cross-linker for 1 h [1 mg/mL, 1 $\times$ PBS (Pierce)]. Cell lysates were prepared [1% Triton X-100, 10% glycerol, 140 mM NaCl, 3 mM MgCl_2 , 50 mM HEPES (pH 7.4), and protease inhibitor mixture (Sigma)], and equivalent amounts of total protein (500 μg) were immunoprecipitated with an anti-ABCA1 antibody or an anti-pan-syntrophin antibody, which recognizes the α 1-, β 1-, and β 2-syntrophin isoforms.^{24,25} The precipitated complexes were reduced and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the resulting gels imaged using a Typhoon Phosphor Imager (GE Healthcare). To assess cell surface ABCA1 levels, cells were chilled on ice for 10 min, washed with ice-cold 1 $\times$ PBS three times, and biotinylated with the non-membrane permeable sulfo-NHS-biotin cross-linker (1 mg/mL) for 1 h. The reaction was quenched with 100 mM glycine; a cell lysate was prepared as described above, and 50 μg of total protein was incubated with 30 μL of NeutrAvidin beads overnight at 4 °C (Pierce). Washed beads were eluted by boiling in running buffer, and the isolated biotinylated proteins were separated by SDS–PAGE and immunoblotted for the presence of ABCA1. ABCA1 protein stability was determined by treating cells with cycloheximide (100

$\mu\text{g/mL}$), and cell lysates were subsequently collected at 1, 2, 4, 6, and 8 h and assessed for ABCA1 protein expression by immunoblotting.

Statistical Analysis

All statistical analyses were performed using a Student's *t* test with a sample size of $n = 3$. A *p* value of <0.05 was considered statistically significant.

Hazardous Procedures

The described experiments utilize cell and molecular procedures involving murine retroviruses carrying oncogenes that must be conducted in a BL-2 cell culture facility. Radioisotopes are used in the lipid efflux assays and must be used in accordance with local regulatory requirements for safe handling and disposal of these materials.

RESULTS

Generation of Immortalized Macrophages from *Abca1*^{-/-} and *Syntrophin* ^{$\alpha 1$ -/- $\beta 2$ -/-} mice

ABCA1 is regulated at the transcriptional, translational, and post-translational levels. Our work indicates protein-protein interactions mediate aspects of this post-translational regulation, and we have shown that ABCA1 binding of the amphipathic helices within apoA-I is necessary but not sufficient for the transfer of lipid to apoA-I.^{11,12} Moreover, using mass spectrometry to identify other factors that were copurified with ABCA1 when expressed in HEK 293 cells, we found three syntrophin isoforms ($\alpha 1$, $\beta 1$, and $\beta 2$) associated with the transporter through its C-terminal cytoplasmic domain.²¹ Although overexpression of the syntrophins stabilizes ABCA1 protein expression and increases efflux activity, a role for the endogenous syntrophins in regulating macrophage efflux is less clear. To address this question, we obtained bone marrow from mice lacking ABCA1 (*Abca1*^{-/-}) or mice lacking both the $\alpha 1$ - and $\beta 2$ -syntrophins (*Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-}). Note, to date, the $\beta 1$ -*syn* locus has not been deleted in mice.²³ Marrow cells were cultured in L929-conditioned medium in the presence of a murine retrovirus carrying the v-myc and v-raf oncogenes to generate immortalized macrophages after the withdrawal of conditioned media. Limiting dilution established single-cell clonal lines that were first compared to primary bone marrow macrophages for expression of ABCA1 protein. Immortalized wild-type cells still expressed comparable levels of the transporter relative to the primary macrophages, while immortalized *Abca1*^{-/-} macrophages, as expected, showed a complete loss of ABCA1 protein (Figure 1A). Flow cytometry shows the immortalized cells continued to express the F4/80 and CD11B cell surface markers, indicating they have retained the macrophage phenotype (Figure 1B). Likewise, when treated with a synthetic LXR agonist, the wild-type line upregulated total and cell surface ABCA1 protein expression (Figure 1C–E), whereas the *Abca1*^{-/-} macrophages did not. Moreover, compared to primary macrophages, the immortalized wild-type line effluxes comparable levels of cholesterol to lipid free apoA-I, whereas the *Abca1*^{-/-} line, and primary macrophages lacking ABCA1, had no apoA-I-dependent efflux (Figure 2A,B). These results indicate the immortalized wild-type macrophages retained regulated ABCA1-dependent apoA-I efflux. Hence, we further established the utility of the lines to study the function of ABCA1 in the process of reverse cholesterol transport by assessing how they transfer cholesterol to mature HDL particles. As opposed to efflux

mediated by lipid poor apoA-I, HDL-dependent efflux has been reported to have a minor dependence upon ABCA1 function compared to the role of ABCG1.⁹ Using 50 $\mu\text{g/mL}$ HDL as an acceptor, we confirmed that at baseline, and when LXR signaling was activated, there was no significant difference in the efflux of cholesterol from cells lacking ABCA1 (Supplementary Figure 1). However, at 10 $\mu\text{g/mL}$ HDL, immortalized *Abca1*^{-/-} cells had a significant deficit in both cholesterol and phospholipid efflux (Figure 2C,D). Given this result, we confirmed that with limiting amounts of HDL, primary bone marrow macrophage efflux also had a significant ABCA1 dependence (Figure 2E,F). Finally, loss of ABCA1 function is reported to exacerbate foam cell formation and inflammatory signaling through effects on toll-like receptors. Thus, we first assessed whether the *Abca1*^{-/-} line showed a stronger tendency to accumulate lipid when exposed to modified LDL particles (acetylated LDL), and if so, was this exacerbated by treatment with the TLR4 ligand, bacterial cell wall lipopolysaccharide (LPS)? Compared to the wild-type line, the *Abca1*^{-/-} cells showed more lipid accumulation at baseline and after treatment with AcLDL, and this phenotype was slightly exacerbated by LPS exposure (Figure 3A,B). Moreover, LPS treatment of the *Abca1*^{-/-} cells was associated with an 11–26-fold higher level of expression of the IL-6 and IL-12 cytokine mRNA and an increased level of expression of cell surface CD14, a TLR4 coreceptor that binds LPS and is critical for signal transduction mediated by this receptor (Figure 3C–E). In aggregate, these results show the immortalized *Abca1*^{-/-} macrophages phenocopy key features of primary bone marrow macrophages lacking ABCA1 and thus are a useful system for studying ABCA1 structure–function relationships.

The ABCA1–Syntrophin Complex Is Induced by LXR Agonists and Can Bind ApoA-I

Given immortalized macrophages maintained ABCA1 function and regulation, these cells were leveraged to investigate the ABCA1–syntrophin protein complex. First, *Abca1*^{+/+} and *Abca1*^{-/-} cells were treated either with apoA-I or with LXR agonists, and the endogenous syntrophins expressed in these cells were isolated with a pan-syntrophin antibody capable of precipitating the three syntrophin isoforms that bind ABCA1. Immunoblotting for ABCA1 in the syntrophin precipitate showed that a small amount of the complex was detectable in untreated wild-type cells, but not *Abca1*^{-/-} cells, and that treatment of the cells with apoA-I or an LXR agonist increased the amount of ABCA1 associated with the syntrophins (Figure 4A, top left panel). Assessment of input lysate ABCA1 and syntrophin protein indicates the increased ABCA1–syntrophin complex largely paralleled increased total ABCA1 protein induced by the treatments, a result that suggests the amount of ABCA1 limits formation of the complex (Figure 4A, right panels). Because the direct interaction between apoA-I and ABCA1 is essential for efflux activity, we tested if the ABCA1–syntrophin complex was capable of directly interacting with apoA-I. Here HEK293 cells transfected with empty vector or with a vector expressing ABCA1, or the *Abca1*^{+/+} and *Abca1*^{-/-} macrophages, were incubated with ¹²⁵I-labeled apoA-I for 1 h at 37 °C. The amount of total apoA-I associated with ABCA1, as well as that associated with the ABCA1–syntrophin complex, was then determined by chilling the cells to 4 °C, exposing the cells to a thiol-reducible chemical cross-linker (DSP) that traps the ABCA1–apoA-I protein complex, and immunoprecipitating either ABCA1 or the syntrophins. After reduction of the cross-links and SDS–PAGE, the amount of radiolabeled apoA-I in the precipitates was determined by phosphor imaging. Consistent with earlier results, overexpressed or endogenous ABCA1

bound apoA-I (Figure 4B, left panel). Likewise, apoA-I was specifically detected in the syntrophin precipitate in the 293ET cells (Figure 4B, right panel). To assess whether the interaction of ABCA1 with apoA-I had a critical dependence upon the $\alpha 1$ - and $\beta 2$ -syntrophins, we analyzed the amount of apoA-I that associates with ABCA1 in the immortalized macrophages derived from *Syntrophin* ^{$\alpha 1$ -/- $\beta 2$ -/-} mice. We confirmed the specificity of the tripartite complex between the ABCA1–apoA-I complex and the syntrophins (Figure 4C, bottom panel) but found that loss of these two syntrophins causes an only minor decrease in the amount of total apoA-I that associates with ABCA1 (Figure 4C, top panel). Thus, ABCA1 can simultaneously bind both apoA-I and the syntrophins, but loss of the $\alpha 1$ - and $\beta 2$ -syntrophins does not profoundly disrupt the physical interaction between ABCA1 and apoA-I.

Loss of $\alpha 1$ - and $\beta 2$ -Syntrophin Disrupts ApoA-I-Dependent Stabilization of Cell Surface ABCA1 and Induces a Pro-inflammatory Phenotype

Because tripartite complex ABCA1 forms with the syntrophins and apoA-I is not essential for the interaction of ABCA1 and apoA-I, this suggests the complex may mediate other signaling events that are transduced by apoA-I. One such event is the stabilization of cell surface ABCA1 mediated by apoA-I. To test if loss of the $\alpha 1$ - and $\beta 2$ -syntrophins disrupts this apoA-I stabilization of ABCA1, we treated the wild-type and *Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-} macrophage lines with apoA-I for increasing times and assessed the amount of cell surface ABCA1 using a membrane impermeable biotin cross-linker followed by capture of biotinylated proteins with NeutrAvidin beads. Probing the biotinylated fraction and the input lysates for ABCA1 showed that treatment of wild-type cells with apoA-I markedly increased the amount of cell surface and total ABCA1, whereas in the *Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-} cells, the ability of apoA-I to increase both cell surface and total ABCA1 was blunted (Figure 5A). To test if this difference in ABCA1 levels was due to altered protein stability in *Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-} macrophages, cycloheximide was used to block protein synthesis. Compared to that in wild-type cells, the level of ABCA1 protein turnover was 50% greater in the *Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-} macrophages (Figure 5B, $t_{1/2}$ of 13.2 ± 1.6 h for the wild type vs 6.5 ± 1.6 h for *Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-}, $n = 3 \pm \text{SD}$, $p = 0.006$). Finally, because cell surface ABCA1 has been reported to inhibit signaling through the TLR receptors, we tested whether loss of the $\alpha 1$ - and $\beta 2$ - syntrophin also causes a pro-inflammatory response to LPS. This was found to be the case in that *Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-} cells upon their exposure to LPS expressed 4-, 8-, and 12-fold more highly than IL-1 β , IL-6, and IL-12 mRNA, respectively, compared to the wild-type cells (Figure 5C). In contrast, message levels of the CD-36 scavenger receptor were not significantly affected by loss of the $\alpha 1$ - and $\beta 2$ -syntrophins.

Loss of the $\alpha 1$ -, $\beta 1$ -, and $\beta 2$ -Syntrophins Does Not Significantly Impact Immortalized or Primary Macrophage ApoA-I-Dependent Efflux

That loss of $\alpha 1$ - and $\beta 2$ - syntrophins produced a pro-inflammatory phenotype, and modulated levels of cell surface ABCA1 suggested these PDZ proteins may play a role in regulating foam cell formation and cholesterol efflux. However, when either the immortalized or primary bone marrow-derived *Syn* ^{$\alpha 1$ -/- $\beta 2$ -/-} macrophages were compared to control cells, no significant differences were observed for cholesterol efflux to either lipid poor apoA-I or HDL (Figure 6A–D and Supplementary Figure 2A). Likewise, primary

Syn ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} macrophages did not exhibit a stronger tendency to form foam cells upon being incubated with modified LDL and LPS (Supplementary Figure 2B). This result was puzzling considering the pro-inflammatory phenotype of the immortalized *Syn* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} macrophages and the lower levels of cell surface ABCA1 detected in these cells. Because we had previously found that a third syntrophin isoform ($\beta 1$) can also bind ABCA1, we considered whether this isoform may be compensating for the loss of the $\alpha 1$ - and $\beta 2$ -syntrophins. Indeed, when the levels of $\beta 1$ -syntrophin mRNA and protein were assessed in primary and immortalized *Syn* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} macrophages, respectively, there was evidence of a compensatory upregulation of this syntrophin isoform (Figure 7A,B). Thus, the upregulation of $\beta 1$ -syntrophin may have compensated for the loss of the other two isoforms. Hence, we tested a series of lentiviral constructs encoding small interfering RNAs targeting the $\beta 1$ -syntrophin message for the ability to suppress expression of $\beta 1$ -syntrophin transiently expressed in 293 cells (data not shown). Viral particles carrying the most effective $\beta 1$ -syntrophin siRNA or control particles expressing a GFP siRNA were then used to transduce primary *Syn* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} bone marrow cultures, and apoA-I-dependent efflux of the derived macrophages was again measured. In spite of a near complete knockdown of $\beta 1$ -syntrophin protein in these experiments, and diminished levels of total ABCA1, we again detected no significant efflux deficit in these cells lacking all three syntrophin isoforms (Figure 7C,D). These results indicate macrophage ABCA1 efflux is not critically dependent upon the ABCA1-syntrophin protein complex.

DISCUSSION

Here we generated immortalized macrophage lines from *Abca1* ^{$^{-/-}$} and *Syntrophin* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} mice. The matched wild-type lines retain robust ABCA1 protein expression and efflux activity, which is sensitive to LXR regulation. Probucol inhibits ABCA1 function in wild-type cells, and loss of ABCA1 ablates efflux to apoA-I, increasing the extent of foam cell formation and pro-inflammatory signaling. Thus, bone marrow macrophage immortalization by retroviral transduction of the v-raf and v-myc oncogenes preserves key aspects of the ABCA1 efflux mechanism. Moreover, the system's utility for probing structure-function relations was demonstrated by analyzing the endogenous protein complex ABCA1 forms with syntrophin PDZ scaffolding factors. We show the ABCA1-syntrophin complex can associate with apoA-I and modulate the cell surface expression of ABCA1, but loss of the three syntrophin isoforms known to bind ABCA1 did not significantly impact *ex vivo* macrophage cholesterol and phosphatidylcholine efflux.

Why then does ABCA1 engage the syntrophins in a protein complex? We confirmed in macrophages, at endogenous expression levels, ABCA1 forms a complex with the syntrophins. Additionally, both Muneira et al. and Albrecht et al. have shown ABCA1 binds syntrophins in central and peripheral nervous tissues.^{19,26} Interestingly, the ABCA1-syntrophin complex in Schwann cells has a polarized distribution that restricts it to the Cajal bands and excludes it from periaxonal regions.²⁶ Likewise, in the liver where ABCA1 function is critical for HDL biogenesis, localization of transporter is also highly polarized to the hepatocyte basolateral membrane²⁷ (and unpublished observations). Given the high level of $\beta 1$ -syntrophin expression in the liver, it may be that interaction between ABCA1 and the syntrophins, or other PDZ proteins, plays a role in the polarized trafficking of ABCA1.^{20,25}

In vivo experiments that suppress $\beta 1$ -syntrophin expression in the *Syntrophin* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} mice will help resolve whether the syntrophins are important in maintaining polarized expression and function of ABCA1 in the liver. Indeed, a recent report assessed ABCA1 expression levels in the liver of the *Syntrophin* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} mice and found lower levels of the transporter; however, this trend did not reach significance, and suppression of the $\beta 1$ -syntrophin was not reported.²⁸ The level of liver apoA-I protein also showed a downward trend, but interestingly, both apoA-I and ABCA1 mRNA levels showed an increasing trend, suggesting post-transcriptional effects leading to a lower level of protein expression. Likewise, in our *Syntrophin* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} macrophages, we found less cell surface and total ABCA1 expression, and this was associated with a more rapid turnover of the transporter. Moreover, *Syntrophin* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} macrophages showed a pro-inflammatory response to LPS, as did the *Abca1* ^{$^{-/-}$} macrophages. Given the cell surface ABCA1 lipid flipping activity is known to alter the recruitment to and activity of TLR receptors in cholesterol rich membrane microdomains, it may be that the syntrophins also effect this process through their interaction with ABCA1.^{29–31} However, given the level of SR-BI expression in the liver of *Syntrophin* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} mice was also found to be markedly reduced, it is also possible syntrophin protein–protein interactions can affect a number of transporters involved in modulating cellular cholesterol levels, lipid microdomains, and efflux potential.³² However, our *Syntrophin* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} macrophages did not have marked changes in SR-BI expression, nor was the amount of accumulated cholesterol different in these cells after exposure to acetylated LDL (Supplemental Figure 2). This indicates scavenger receptor function remained intact in the *Syntrophin* ^{$\alpha 1^{-/-}/\beta 2^{-/-}$} macrophages. Likewise, we show that loss of the protein complex ABCA1 forms with the syntrophins in primary macrophages has little or no impact on lipid efflux to apoA-I, even when expression of $\beta 1$ -syntrophin, the third isoform known to bind the ABCA1 C-terminus, is suppressed with siRNAs. Thus, how the syntrophins can modulate ABCA1 protein stability without an impact on efflux remains to be determined. Given reports that it is dimers of ABCA1 that are active in the efflux cholesterol,³³ it may be that the syntrophins preferentially bind a pool of inactive monomeric ABCA1. When this interaction is disrupted, the stability of the monomeric pool is reduced but there is a compensatory increase in the level of active dimeric ABCA1, and hence, these effects cancel out in terms of net cellular efflux activity. Such a possibility is diagramed in Figure 7E. It may be that testing more efflux parameters, such as the time that the cells are equilibrated with the radiolabeled cholesterol or how long they are exposed to the acceptors, may detect a more nuanced effect of the syntrophins on efflux activity.³²

In summary, we show that immortalization of bone marrow-derived macrophages with a J2 retrovirus carrying the v-raf and v-myc oncogenes generates cell lines that retain robust ABCA1 expression that is sensitive to LXR agonism and that efflux cholesterol and phospholipid in an apoA-I- and HDL- dependent manner. In addition to structure–function studies, the cell lines we have generated should have further utility in basic and translational studies of reverse cholesterol transport. Indeed, Khera et al., along with other laboratories, have used the J774 murine line to profile sera samples from patients with cardiovascular disease to show that cholesterol efflux capacity of the sera is inversely correlated with carotid intima-media thickness, the presence of plaque, and incident cardiovascular events.^{34–36} Now, our lines can be used to differentiate efflux parameters and disease states

that have a specific dependence on ABCA1 function. Likewise, it will be of interest to generate lines lacking ABCG1 to assess the contribution this transporter may have in predicting efflux capacity and cardiovascular disease risk.^{37,38} Given outcome studies are longitudinal in nature with variable patient recruitment rates, it is important to standardize assays on well-characterized cell lines with defined genetic ablations, thus allowing for multicenter and multiyear trials. With the cell lines described here, it will now be possible to interrogate how disease states and therapies that modulate HDL composition impact ABCA1-dependent macrophage cholesterol efflux.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was funded by grants to M.L.F. from the American Heart Association (09GRNT2260352) and the National Institutes of Health (HL074136, HL101274, and HL112661).

ABBREVIATIONS

ABCA1	ATP cassette transporter A-1
HDL	high-density lipoprotein
LDL	low-density lipoprotein
LPS	lipopolysaccharide
DSP	dithiobis(succinimidyl propionate)
SD	standard deviation

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Figure 1.

v-raf and v-myc immortalized macrophages express ABCA1 in a LXR-regulated manner. Bone marrow-derived macrophages from *Abca1*^{+/+} and *Abca1*^{-/-} mice were immortalized by viral transduction of the v-raf and v-myc oncogenes. (A) The immortalized lines express similar levels of ABCA1 protein when compared to that of primary bone marrow-derived macrophages as determined by immunoblots for ABCA1 and β -actin. (B) The immortalized lines retain uniform and robust expression of the F4/80 and CD11b markers of macrophage differentiation as determined by flow cytometry and increase the level of ABCA1 protein expression in response to treatment with the synthetic LXR nuclear hormone agonist TO-1317 (10 μ m, 24 h) as indicated by immunoblots (C), cell surface immunofluorescent staining (D), and flow cytometry for ABCA1 (E).

Figure 2.

Immortalized *Abca1*^{-/-} macrophages show a complete ablation in apoA-I-dependent efflux and a partial defect in HDL-dependent efflux. (A and B) Cholesterol efflux to apoA-I (10 μ g/mL) by primary and immortalized *Abca1*^{+/+} bone marrow-derived macrophages is similar and stimulated by the LXR agonist TO-901317 (10 μ M), whereas the corresponding *Abca1*^{-/-} cells show no apoA-I-dependent cholesterol efflux. (C and D) Immortalized *Abca1*^{-/-} macrophages show a partial defect in both cholesterol and phospholipid efflux to mature HDL (10 μ g/mL). (E and F) Primary *Abca1*^{-/-} macrophages show a partial defect in both cholesterol and phospholipid efflux to mature HDL (10 μ g/mL). Results of assays performed in triplicate are representative of two or more independent experiments ($n = 3$, $\pm$ SD, * $p < 0.05$, ** $p < 0.01$ compared to control, or HDL-treated samples).

Figure 3.

Immortalized *Abca1*^{-/-} macrophages readily form foam cells and have a pro-inflammatory phenotype. (A) Immortalized *Abca1*^{-/-} macrophages accumulate greater stores of neutral lipid at baseline and after incubation with LPS (1 ng/mL) and acLDL (15 μ g/mL) for 48 h as determined by Oil Red O staining and light microscopy (A) and enzymatic quantification of total cholesterol (B) ($n = 3$, $\pm$ SD, * $p < 0.05$ compared to matched *Abca1*^{+/+} samples). (C and D) Immortalized *Abca1*^{-/-} macrophages treated with LPS (1 ng/mL) for the indicated times express significantly more IL-6 and IL-12p40 mRNA relative to *Abca1*^{+/+} immortalized macrophages ($n = 3$, $\pm$ SD, * $p < 0.05$ compared to matched *Abca1*^{+/+} samples). (E) Immortalized *Abca1*^{-/-} macrophages treated with LPS (1 ng/mL, 24 h) express more cell surface CD14 as determined by flow cytometry.

Figure 4.

ABCA1-syntrophin protein complex that increased in an LXR-dependent manner and can bind apoA-I. (A) Immortalized *Abca1*^{+/+} and *Abca1*^{-/-} macrophages were treated with apoA-I (10 μ g/mL) or LXR agonist TO-901317 (10 μ M) for 16 h, and the amount of ABCA1-syntrophin protein complex was determined by immunoprecipitation using an anti-syntrophin antibody. The amount of ABCA1 and syntrophin captured in the precipitates (left) and in the input lysates (right) was determined by immunoblotting. (B) Endogenous syntrophins expressed in 293ET cells can bind the apoA-I-ABCA1 protein complex. ¹²⁵I-labeled apoA-I was incubated with 293ET cells transiently transfected with ABCA1 cDNA or with the immortalized *Abca1*^{+/+} and *Abca1*^{-/-} macrophages for 1 h at 37 °C, followed by exposure to a thiol-reducible chemical cross-linker. Total levels of the apoA-I-ABCA1 complex were assessed by immunoprecipitation of ABCA1, and the amount of the apoA-I-ABCA1 complex associated with the syntrophins was assessed by immunoprecipitation with a pansyntrophin antibody. Shown are phosphor images of coprecipitated [¹²⁵I]apoA-I. (C) Formation of the apoA-I-ABCA1 complex does not critically depend upon the β 2- and α 1-syntrophins as determined by analysis of binding of [¹²⁵I]apoA-I to ABCA1 in the immortalized *Syn* ^{α 1^{-/-} β 2^{-/-} macrophages.}

Figure 5.

Loss of $\alpha 1$ - and $\beta 2$ -syntrophin disrupts apoA-I-dependent stabilization of cell surface ABCA1 and induces a pro-inflammatory phenotype. (A) Stabilization of total and cell surface ABCA1 by apoA-I is disrupted in immortalized *Syn* ^{$\alpha 1^{-/-}\beta 2^{-/-}$} macrophages. Wild-type and *Syn* ^{$\alpha 1^{-/-}\beta 2^{-/-}$} macrophages were incubated with apoA-I (10 μ g/mL) for the indicated time periods followed by exposure to a cell impermeable biotinylated cross-linker. Biotinylated proteins were captured by a NeutrAvidin column, separated by SDS-PAGE, and probed for the amount of ABCA1 by immunoblotting (bottom). Bottom panels show total ABCA1, syntrophin, and β -actin in the input lysates. (B) The level of ABCA1 protein turnover is increased in immortalized *Syn* ^{$\alpha 1^{-/-}\beta 2^{-/-}$} macrophages compared to wild-type cells after inhibition of protein synthesis with cycloheximide ($t_{1/2}$ of 13.2 ± 1.6 h for the wild type vs 6.5 ± 1.6 h for *Syn* ^{$\alpha 1^{-/-}\beta 2^{-/-}$} , $n = 3 \pm \text{SD}$, $p = 0.006$). (C) Immortalized *Syn* ^{$\alpha 1^{-/-}\beta 2^{-/-}$} macrophages display a pro-inflammatory phenotype in response to LPS treatment by expressing more mRNA for the IL-6, IL-12, and IL- β cytokines but not for the CD-36 scavenger receptor as determined by RT-QPCR assays ($n = 3$, $\pm \text{SD}$, $*p < 0.05$ compared to matched wild-type samples).

Figure 6.

Immortalized and primary $Syn^{\alpha 1-/-\beta 2-/-}$ macrophages display normal lipid efflux. Cholesterol efflux (A and B) and choline-containing phospholipid efflux (C and D) measured in immortalized and primary $Syn^{\alpha 1-/-\beta 2-/-}$ macrophages either at baseline or after stimulation with LXR agonist TO-901317 (10 μM , $n = 3$, $\pm\text{SD}$).

Figure 7. siRNA repression of $\beta 1$ -syntrophin in primary $Syn^{\alpha 1-/-}\beta 2-/-$ macrophages does not affect lipid efflux to apoA-I. Levels of $\beta 1$ -syntrophin mRNA (A) and protein (B) are increased in $Syn^{\alpha 1-/-}\beta 2-/-$ macrophages. (C) Primary $Syn^{\alpha 1-/-}\beta 2-/-$ macrophages expressing a lentivirus-delivered shRNA targeting $\beta 1$ -syntrophin show normal efflux compared to controls transduced with shRNA-targeting GFP ($n = 3$, $\pm$ SD). (D) Immunoblot of the pooled cell lysate from the efflux experiment shown in panel C indicating $\beta 1$ -syntrophin protein was effectively suppressed. (E) Model of the ABCA1-syntrophin interaction that assumes it increases a pool of stable monomeric ABCA1 that can directly interact with apoA-I but is not active for lipid efflux. Hence, when disrupted, although the level of total ABCA1 protein is reduced, the equilibrium is shifted toward pools of ABCA1 in the monomeric and dimeric states that are active for lipid efflux, and the overall efflux activity of the cell remains unchanged.