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The Macrophage Cholesterol Exporter ABCA1 Functions as an Anti-inflammatory Receptor*

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ATP-binding cassette transporter A1 (ABCA1) is a cell membrane protein that exports excess cholesterol from cells to apolipoprotein (apo) A-I, the major protein in high density lipoproteins. Genetic studies have shown that ABCA1 protects against cardiovascular disease. The interaction of apoA-I with ABCA1 promotes cholesterol removal and activates signaling molecules, such as Janus kinase 2 (JAK2), that optimize the lipid export activity of ABCA1. Here we show that the ABCA1-mediated activation of JAK2 also activates STAT3, which is independent of the lipid transport function of ABCA1. ABCA1 contains two candidate STAT3 docking sites that are required for the apoA-I/ABCA1/JAK2 activation of STAT3. The interaction of apoA-I with ABCA1-expressing macrophages suppressed the ability of lysopolysaccaride to induce the inflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α, which was reversed by silencing STAT3 or ABCA1. Thus, the apoA-I/ABCA1 pathway in macrophages functions as an anti-inflammatory receptor through activation of JAK2/STAT3. These findings implicate ABCA1 as a direct molecular link between the cardioprotective effects of cholesterol export from arterial macrophages and suppressed inflammation.

Two major processes that initiate the formation of atherosclerotic lesions in the artery wall are inflammation and the deposition of excess cholesterol in macrophages. It is believed that both of these events are in response to trapping of sterol-rich lipoproteins in the artery, where they undergo oxidation and other modifications to become inflammatory stimuli that recruit and activate macrophages (1). These cells ingest and degrade the modified lipoproteins, leading to intracellular accumulation of cholesterol ester lipid droplets.

Population studies have shown an inverse relationship between circulating levels of HDLs and risk for cardiovascular disease, implying that factors associated with HDL metabolism are cardioprotective. One of these factors is ABCA1, which exports cholesterol and phospholipids from cells to lipid-poor apoA-I to generate precursors for HDL particles (2). Because it is highly induced by sterols through nuclear receptors, ABCA1 is expressed in cholesterol-loaded cells, such as macrophages in atherosclerotic lesions (3). Loss-of-function mutations in ABCA1 accelerate atherosclerosis (4), which is likely to be the result of enhanced accumulation of cholesterol-rich macrophages in arteries and the hyper-inflammatory responses of these cells.

The cholesterol export function of ABCA1 occurs by a cascade of events involving direct binding of apoA-I to ABCA1, activation of signaling pathways, and solubilization of cholesterol and phospholipid domains formed by ABCA1 on the cell surface (5). We reported previously that incubating ABCA1-transfected baby hamster kidney (BHK)[2] cells with apoA-I or its synthetic mimetic peptides for only minutes dramatically increased autophosphorylation and thus activation of JAK2 by an ABCA1-dependent mechanism. Activation of JAK2 enhanced the apoA-I binding activity of ABCA1 responsible for lipid removal (6, 7). Thus JAK2 plays a feed-forward mechanism for optimizing the lipid export function of ABCA1.

Receptor-mediated activation of JAK2 usually stimulates signaling pathways that activate transcription factors called STATs (8). We therefore investigated the possibility that the interaction of apoA-I with ABCA1 also activates one or more STATs. We found that exposing ABCA1 expressing cells to apoA-I increased phosphorylation of STAT3 without affecting STAT1 or STAT5, and this was independent of the lipid export activity of ABCA1. Because STAT3 plays a major role in suppressing inflammatory cytokine production by macrophages (9), a cell-type that expresses high levels of ABCA1 when sterol loaded, we also examined the possibility that the interaction of apoA-I with ABCA1 could suppress macrophage inflammatory cytokine production through STAT3. Results show that incubating apoA-I with activated ABCA1-expressing macrophages suppresses production of inflammatory cytokines IL-1β, IL-6, and TNF-α by a STAT3-dependent process, raising the possibility that the ABCA1 has a direct anti-inflammatory function in addition to its lipid export activity.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—STAT3, phospho-STAT3, and phospho-JAK2 antibodies were purchased from Cell Signaling; and STAT1, phopsho-STAT1, anti-STAT5, phospho-STAT5, and JAK2 antibodies were purchased from Santa Cruz. ABCA1 antibody was purchased from Novus. The JAK2 specific inhibitor AG490 was purchased from Sigma, and cell-per-

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[2] The abbreviations used are: BHK, baby hamster kidney; JAK, Janus kinase; STAT, signal transducers and activators of transcription; IL, interleukin; TNF, tumor necrosis factor; HDL, high density lipoprotein; LDL, low density lipoprotein; WT, wild-type; DMEM, Dulbecco's modified Eagle's medium; siRNA, small interfering RNA; RT, reverse transcription; LPS, lipopolysaccharide; P-STAT, phosphorylated STATs; NBD, nucleotide binding domain.

meable STAT3 inhibitor was purchased from Calbiochem. ApoA-I was purified from HDL as described previously (10).

Mice—C57BL/6 and DBA mice were purchased from Jackson Laboratories. ABCA1$^{-/-}$/DBA mice were a gift from Robert Aiello, Pfizer-Wyeth; STAT3$^{flox/flox}$/C57BL/6 mice were a gift from Shizuo Akiro, Osaka University; and Lys-M-Cre/C57BL/6 mice were a gift from Karin Bornfeldt, University of Washington. To generate mice lacking STAT3 in macrophages and neutrophils, STAT3$^{flox/flox}$ were crossed with Lys-M-Cre mice to generate mice that carried the homozygous STAT3$^{flox/flox}$/Lys-M-Cre mice. Control wild-type mice were generated by interbreeding STAT3$^{wt/wt}$ with Lys-M-Cre mice.

ABCA1-transfected Cells and Cell Culture—Wild-type and mutant BHK cells expressing mifepristone-inducible human ABCA1 were generated as described previously (11). Control (mock) BHK cells were derived from the same clonal line transfected with plasmids lacking the ABCA1 cDNA insert. Site-directed mutagenesis of *ABCA1* was carried out using the QuikChange XL Site-directed mutagenesis kit (Stratagene, CA) (5). Cells were selected against Neozi, and clonal lines expressing similar amounts of wild-type (WT) ABCA1, mutants Y924F, Y1990F, or Y924/1990F were used for the experiments. BHK cells were maintained in DMEM with 10% fetal bovine serum until experiments. J774 cells were cultured in DMEM with 10% fetal bovine serum. To induce ABCA1 expression, J774 cells were loaded overnight with 50 μg/ml acetylated LDL in the presence of 5 μM TO901317 (an L*X*R agonist that induces ABCA1) in DMEM containing 0.1% fatty acid-free bovine albumin (12). Thioglycollate-elicited peritoneal macrophages were obtained as previously described (13) and cultured in DMEM with 10% fetal bovine serum for 2 h, unseeded cells were washed away, and the medium was changed to DMEM, 0.1% fatty acid-free bovine albumin. To induce ABCA1 expression, cells were loaded with 50 μg/ml acetylated LDL overnight. Because non-induced macrophages or BHK cells express very low or no detectable endogenous ABCA1, cells treated with inducers are defined as "cells expressing ABCA1." For cytokine expression experiments, cells were pre-treated with or without 10 μg/ml of apoA-I in DMEM, 0.1% fatty acid-free bovine albumin for 3 h, washed twice, treated with or without 10 ng/ml LPS for 3 h, and then processed for RNA extractions. Bone marrow macrophages for immunoblots were derived by flushing out mouse femurs and tibias with RPMI 1640, 10% fetal bovine serum and culturing cells for 1 week.

Cholesterol Export—Cellular cholesterol was labeled with 1 μCi/ml of [^{3}H]cholesterol (PerkinElmer Life Sciences) added to the growth medium 24 h prior to mifepristone incubations. Washed cells were then incubated with DMEM/BSA minus or plus 10 μg/ml apoA-I, and medium and cells were assayed for free [^{3}H]cholesterol (14). ApoA-I-mediated cholesterol efflux was calculated as the percent total [^{3}H]cholesterol released into medium after subtraction of values obtained in the absence of apoA-I and was normalized to values for cells expressing wild-type ABCA1. Cells without induced ABCA1 had cholesterol efflux values insignificantly above induced cells incubated in the absence of apoA-I.

Immunoblots and Immunoprecipitation—Cells were lysed in Tris-HCl buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 1% Nonidet P-40) supplemented with protease inhibitors (Complete mini; Roche) and phosphatase inhibitors (phosphatase inhibitor mixture II; Calbiochem) and then centrifuged at 15,000 $\times$ *g* for 10 min at 4 °C. Protein concentration was measured using the Bio-Rad protein assay reagent as instructed. Equal amounts of protein were added per gel lane, resolved by SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. For immunoprecipitation, 1000–2000 μg of protein of 1 ml of lysates were incubated with the indicated antibodies (2–4 μg) overnight at 4 °C followed by incubation for 1 h with Protein A-magnetic beads. Immunocomplexes were then resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Band intensity was quantified from the autoradiograph scans using OptiQuant computer software (Packard Instruments).

siRNA—Thioglycollate-elicited mouse peritoneal macrophages or J774 cells were plated in 24-well plates and maintained in DMEM with 10% (v/v) heat-inactivated fetal bovine serum, penicillin, and streptomycin. Silencer negative control siRNA and silencer validated STAT3 siRNAs (mSTAT3 siRNA: sense, 5′-AAGGCCGTGGTGCGTGAGAAA-3′) were purchased from Ambion. Transfection with siRNA diluted in Opti-MEM I at a concentration of 100 nmol was performed using Hiperfect transfection reagent (Qiagen) according to their instruction. One day after transfection, medium was changed, and cells were loaded overnight with 50 μg/ml of acetylated LDL in DMEM without (peritoneal macrophages) or with 5 μM TO901317 (J774 macrophages). Cells were treated with or without 10 μg/ml of apoA-I for 3 h, washed twice, and then treated with/without 10 ng/ml of LPS for 3 h. Cells were collected for RT-PCR analyses.

RT-PCR—The one-step real-time RT-PCR was performed to quantify cytokines mRNA expression using the Mx4000 Muliplex Quantitative PCR system (Stratagene, La Jolla, CA) and the TaqMan one-step RT-PCR master reagent kit (ABI), according to the instructions. The amplifications of cytokines and glyceraldehyde-3-phosphate dehydrogenase RNA were run using 100 ng of total RNA retrieved from samples using the Agilent total RNA isolation kit (Agilent Technology). Reactions were prepared in a 96-well optical grade PCR plate in a total of 50 μl containing the following components: 100 ng of total RNA, 25 μl of 2$\times$ master mix, 2 units of the reverse transcriptase SuperScript II, and 25 μl of TaqMan Gene Expression assay mixture (including primers and probe, from ABI). Thermal cycling conditions consisted of an initial reverse transcription step at 45 °C for 30 min, followed by 10 min at 95 °C, and then followed by 40 cycles of amplification. Each cycle of amplification consisted of a denaturizing step at 95 °C for 30 s and an annealing/extension step at 60 °C for 1 min. The IL-1β, TNF-α, IL-6, glyceraldehyde-3-phosphate dehydrogenase, and 18S primers and probes used for the RT-PCR were purchased from Applied Biosystems and used TaqMan Gene Expression Assays.

Data Analyses—Results were calculated as mean $\pm$ S.D. of 3–6 values, and statistical differences were calculated by Student's *t* test. Differences were considered to be significant at $p < 0.05$.

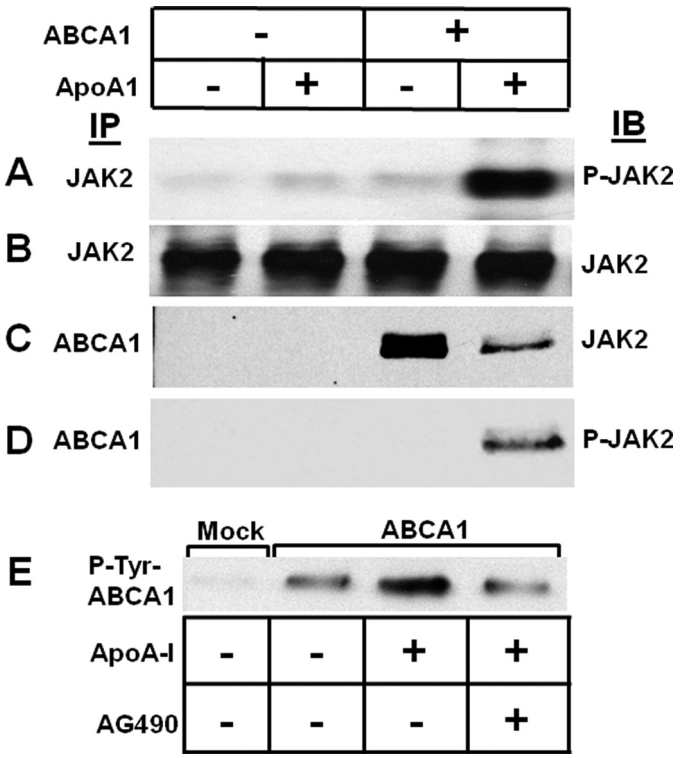

FIGURE 1. **ApoA-I stimulates ABCA1-bound JAK2 autophosphorylation and ABCA1 tyrosine phosphorylation.** ABCA1-transfected BHK cells were treated without (−, *Mock*) or with (+, *ABCA1*) 10 nM mifepristone to induce ABCA1; cells were treated without (−) or with (+) 10 μg/ml of apoA-I for 15 min (*A–D*) or 1 h (*E*); JAK2 (*A* and *B*) or ABCA1 (*C–E*) were immunoprecipitated from lysates; proteins were resolved by SDS-PAGE and transferred to nitrocellulose; and P-JAK2 (*A* and *D*), total JAK2 (*B* and *C*), or phosphotyrosine residues (*E*) were detected by immunoblot analyses. To block JAK2-mediated phosphorylation (*E*), cells were pre-treated with AG490 (+) for 30 min prior to the apoA-I incubations.

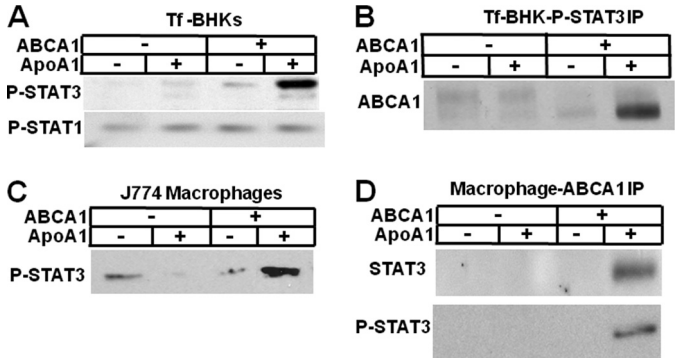

FIGURE 2. **apoA-I interactions with ABCA1-expressing cells increases phosphorylation of ABCA1-associated STAT3.** ABCA1-nonexpressing (−, untreated) and -expressing (+, induced) transfected BHK cells (*Tf-BHKs*, induced with mifipristone) (*A* and *B*) or J774 macrophages (*C* and *D*, induced with the LXR agonist TO901317) were incubated for 1 h without (−) or with (+) 10 μg/ml of apoA-I; and whole cell lysates (*A* and *C*) or immunoprecipitated P-STAT3 (*B*) or ABCA1 (*D*) were immunoblotted for phosphorylated STAT3 (P-STAT3) (*A* and *C*), phosphorylated STAT1 (P-STAT1) (*A*), ABCA1 (*B*), whole STAT3 (*D*), or P-STAT3 (*D*). Total STAT3 in BHK cells and J774 macrophages did not change under any treatment conditions. ABCA1 was undetectable in the absence of inducer.

RESULTS

Using an antibody specific for the phosphorylated form of JAK2 (P-JAK2), we found that incubating ABCA1-transfected BHK cells with apoA-I for only minutes dramatically increased phosphorylation and thus activation of JAK2, which was not observed in cells lacking ABCA1 (Fig. 1*A*). The total cell content of JAK2 was unaffected by either ABCA1 expression or exposure to apoA-I (Fig. 1*B*).

We carried out co-immunoprecipitation studies to determine whether JAK2 and ABCA1 form a molecular complex. When ABCA1 was induced in transfected BHK cells, JAK2 co-immunoprecipitated with ABCA1 whether or not cells were treated with apoA-I (Fig. 1*C*). This JAK2 was phosphorylated only when the ABCA1-expressing cells were treated with apoA-I (Fig. 1*D*). We also found that treating lysates of ABCA1-expressing BHK cells with a P-JAK2 antibody co-precipitated ABCA1 (not shown). Thus, JAK2 constitutively binds to ABCA1, where it is activated by apoA-I.

Because JAK2 is a tyrosine kinase, we examined ABCA1 immunoprecipitated from transfected BHK cells for changes in its phosphotyrosine content using an antibody specific for this modified amino acid. Phosphorylated tyrosines were detected in ABCA1 in the basal state, but this increased over 2-fold when cells were incubated with apoA-I (Fig. 1*E*). Reversal of this increase by AG490 (a JAK2-specific inhibitor) implied that JAK2 was responsible for this effect. These results are consistent with the idea that the interaction of apoA-I with ABCA1-expressing cells rapidly activates JAK2, which in turn phosphorylates ABCA1 tyrosines.

Activation of JAK2 through binding of ligands to a wide variety of plasma membrane receptors also activates one or more of a family of transcription factors called STATs (8). We therefore tested the possibility that the interaction of apoA-I with ABCA1 activates a STAT using antibodies specific for different P-STATs. Incubating BHK cells lacking ABCA1 with apoA-I had no effect on phosphorylation of STAT3, STAT1 (Fig. 2*A*), or STAT5 (not shown). When ABCA1 was induced, however, apoA-I stimulated STAT3 but not STAT1 (Fig. 2*A*) or STAT5 phosphorylation. This effect was inhibited by AG490 (not shown), implying that it was mediated by JAK2. When lysates of transfected BHK cells were treated with an antibody specific for P-STAT3, ABCA1 was co-immunoprecipitated with P-STAT3 only from cells expressing ABCA1 and incubated with apoA-I (Fig. 2*B*).

We also examined the possibility that apoA-I-mediated activation of STAT3 occurs in cholesterol-loaded macrophages, the cell-type most relevant to ABCA1 cell biology *in vivo* (3). P-STAT3 levels were markedly increased in J774 macrophages when they were incubated with the LXR agonist TO901317 to induce ABCA1 and then treated with apoA-I (Fig. 2*C*). Total STAT3 and P-STAT3 were co-immunoprecipitated with ABCA1 only from macrophages that were induced to express ABCA1 and exposed to apoA-I (Fig. 2*D*). Thus, incubating ABCA1-expressing macrophages with apoA-I recruits STAT3 to ABCA1 where it becomes phosphorylated. Taken together, these results suggest that the interaction of apoA-I with ABCA1-expressing cells rapidly activates JAK2, which creates phosphorylated docking sites on ABCA1 that bind and activate STAT3.

The STAT3 docking sites in cytosolic domains of plasma membrane receptors have a highly conserved Y*XX*Q sequence (15). We searched ABCA1 for these motifs and found one in

each of the large cytosolic loops that contain the nucleotide binding domains (NBDs): at amino acids 924–927 (YEGQ) in NBD1 and residues 1990–1994 (YCPQ) in NBD2. These motifs are highly conserved between ABCA1s of different species (chimpanzee, horse, dog, cow, mouse, rat, chicken, zebrafish, zebra finch, platypus, and tree shrew), and the Tyr-1990 motif is conserved between all ABCAs.

We generated cells expressing ABCA1 with phenylalanines substituted for tyrosines in each or both of these Y*XX*Q motifs and selected clonal cell lines that had similar ABCA1-dependent cholesterol efflux activities and ABCA1 protein levels (based on immunoblots) as cells expressing wild-type ABCA1 (Fig. 3*A*). We compared the ability of apoA-I to stimulate phosphorylation of STAT3 in these different cell lines by scanning autoradiograms of blots probed with P-STAT3 antibody and reprobed with total STAT3 antibody and by calculating the ratios.

Exposing apoA-I to BHK cells transfected with wild-type ABCA1 tripled the fraction of STAT3 that was phosphorylated (Fig. 3*B*). Mutating Tyr-924 slightly decreased this fraction, but mutating Tyr-1990 markedly decreased apoA-I-stimulated phosphorylation of STAT3. Mutating both Tyr-924 and Tyr-1990 completely abolished apoA-I-stimulated STAT3 phosphorylation, suggesting some cooperativity between these two sites. Probing immunoprecipitates of STAT3 with an ABCA1 antibody revealed that the apoA-I-stimulated interaction of ABCA1 with STAT3 was completely abolished by the Y924F/Y1990F mutations (Fig. 3*C*). These findings implicate these motifs, especially Tyr-1990, as the docking sites on ABCA1 required for STAT3 activation.

A lack of effect of the Y924F and Y1990F mutations on cholesterol efflux despite a loss of STAT3 phosphorylation (Fig. 3, *A* and *B*) indicates that the ABCA1-dependent activation of STAT3 plays no role in the cholesterol export function of ABCA1. This idea was further supported by studies showing that silencing of STAT3 with a siRNA had no effect of apoA-I-mediated cholesterol or phospholipid efflux (not shown).

ABCA1 is induced by sterol-sensing L*X*R nuclear receptors and thus is expressed at high levels in cells that accumulate cholesterol, such as tissue macrophages. In macrophages, the JAK2/STAT3 signaling pathway has an anti-inflammatory function (9). We therefore tested the effects of apoA-I on expression of anti-inflammatory cytokines by ABCA1-expressing J774 macrophages. For these studies, we loaded cells with cholesterol and treated them with the LXR agonist TO901317 to induce ABCA1 (16), incubated them without or with apoA-I for 3 h, incubated them for an additional 3 h without or with the inflammatory agent LPS in the absence of apoA-I, and measured the levels of TNF-α, IL-1β, and IL-6 mRNA. The purpose of these studies was to determine whether preincubating ABCA1-expressing macrophages with apoA-I affects subsequent cytokine production in response to LPS.

In agreement with studies by others (17), we found that pretreating these cells with the LXR agonist alone markedly suppressed the ability of LPS to induce TNF-α, IL-1β, and IL-6 (not shown). Despite this baseline suppression, however, exposing cells to LPS for 3 h still increased transcription of these cytokines by 2–4-fold (Fig. 4*A*). Although apoA-I had little effect on cytokine mRNA levels without LPS treatment, it markedly reduced the stimulatory effects of LPS (Fig. 4*A*). Thus, the interaction of apolipoproteins with ABCA1-expressing macrophages suppresses LPS-induced inflammatory cytokine production.

We used siRNA transfections to determine whether apoA-I is exerting its anti-inflammatory effects through STAT3. Results from three experiments showed that transfecting J774 macrophages with STAT3 siRNA reduced STAT3 protein levels by 50–60% when compared with cells transfected

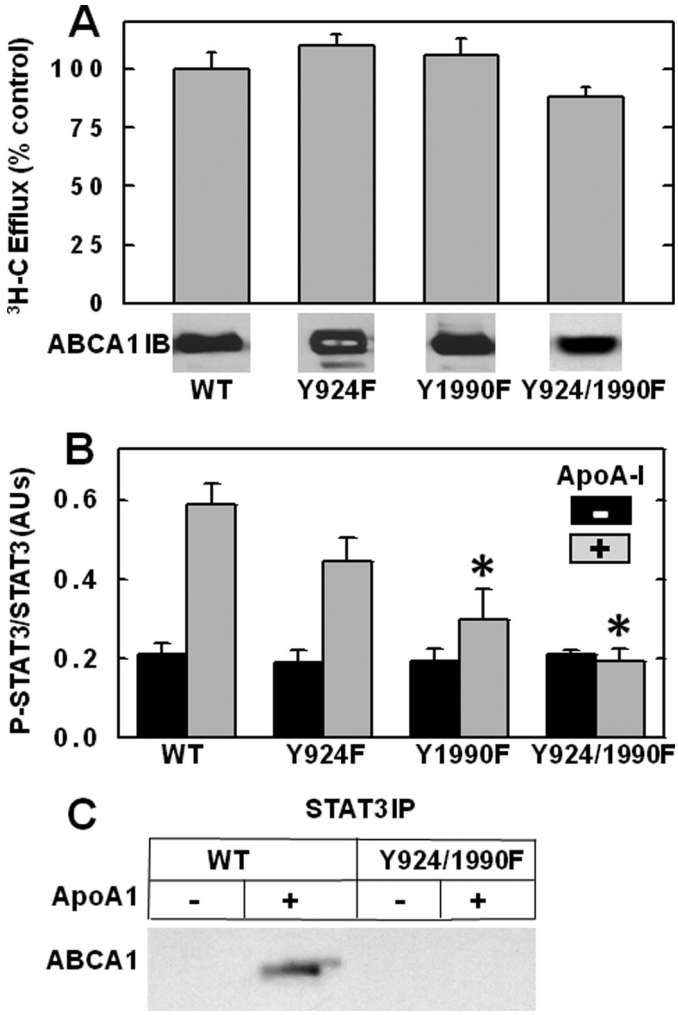

FIGURE 3. **Mutating candidate STAT3 docking sites in ABCA1 reduces apoA-I-stimulated STAT3 phosphorylation and ABCA1 association without affecting cholesterol efflux.** *A*, mifepristone-treated BHK cells transfected with wild-type (*WT*) ABCA1 or ABCA1 containing Y924F, Y1990F, or Y924F/Y1990F substitutions were labeled with [^{3}H]cholesterol and incubated for 2 h without or with 10 μg/ml apoA-I, and cholesterol efflux was calculated as the apoA-I-mediated release of [^{3}H]cholesterol into the medium normalized for values for WT cells. *A*, total ABCA1 protein was detected by immunoblots of ABCA1 from equal amounts of total cell protein from WT and mutant cells resolved on the same gel. *B*, lysates from equal amounts of whole cell protein from BHK cell lines incubated for 1 h without or with 10 μg/ml apoA-I were immunoblotted first with a P-STAT3 antibody and reprobed with a STAT3 antibody. Autoradiograms were scanned and quantified, and the relative amount of STAT3 phosphorylated was calculated as a ratio of P-STAT3 to STAT3 signal for each sample (mean $\pm$ S.D. from four experiments). Total STAT3 did not differ between wild-type and mutant cell lines (not shown). *C*, whole cell lysates from BHK cells transfected with WT and Y924F/Y1990F were immunoprecipitated with anti-STAT3 antibody and immunoblotted with anti-ABCA1 antibody.

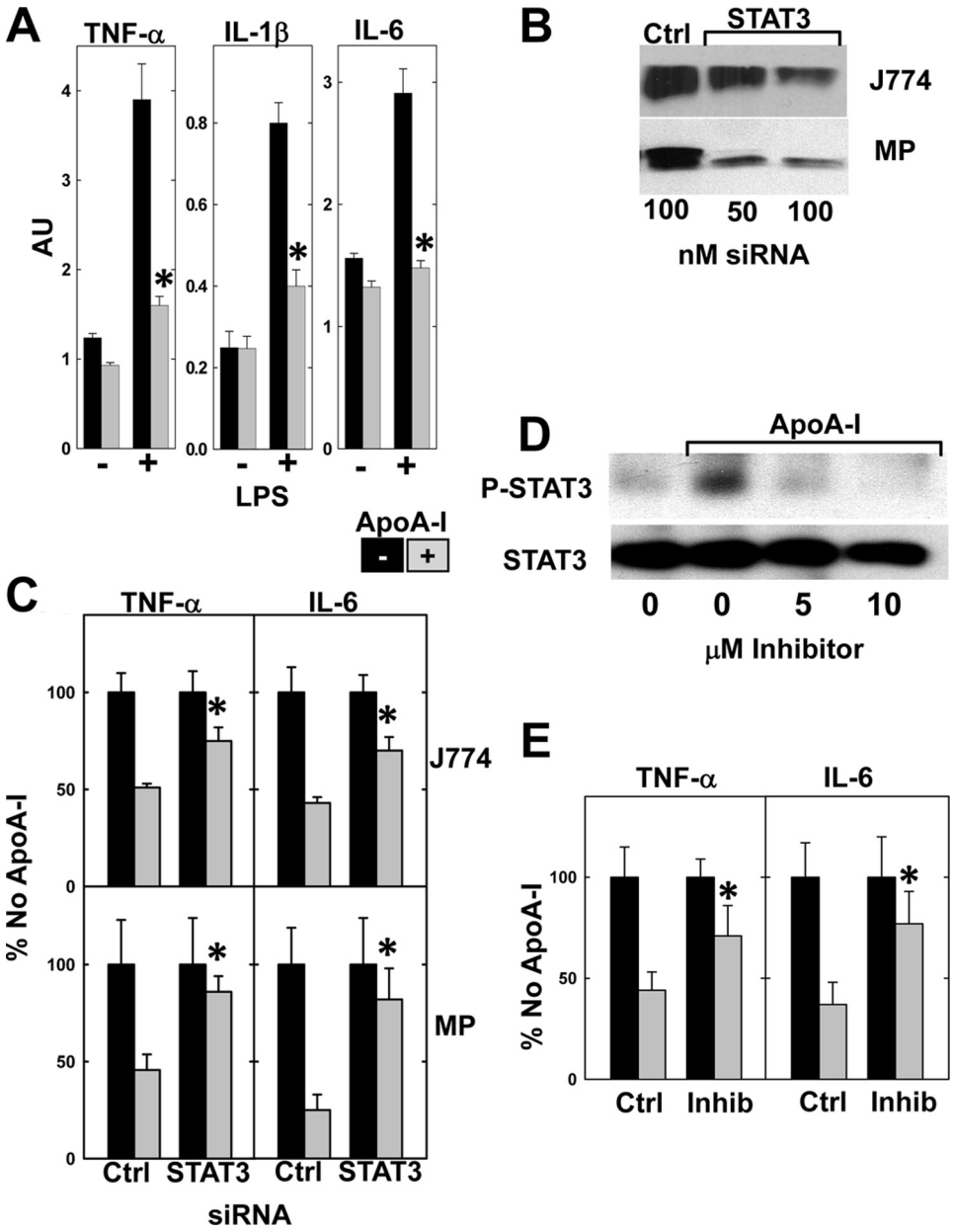

FIGURE 4. **ApoA-I inhibits inflammatory cytokine production in macrophages by a STAT3-dependent mechanism.** *A*, J774 macrophages were loaded for 24 h with 50 μg/ml of acetyl-LDL-derived cholesterol, incubated for 24 h with 5 μg/ml of TO901317 to induce ABCA1, incubated without or with 10 μg/ml of apoA-I for 3 h, and treated without or with 10 ng/ml of LPS for 3 h. TNF-α, IL-1β, and IL-6 mRNA levels were measured by RT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels. Results are mean ± S.D. of triplicate values from 2 separate experiments representative of four similar experiments. *, $p < 0.001$compared with no apoA-I. *AU*, arbitrary units (*B*) J774 (*top*) or mouse peritoneal (*MP*, *bottom*) macrophages were loaded with 50 μg/ml of acetyl-LDL-derived cholesterol, transfected with either a scrambled control (*Ctrl*, 100 nM) or STAT3 siRNA (50–100 nM), and J774 macrophages were incubated for 24 h with 5 μM TO901317. Cells were incubated for 3 h without or with 10 μg/ml of apoA-I, washed, and incubated for 3 h with 10 ng/ml of LPS; and STAT3 levels were measured by immunoblot analyses. *C*, cholesterol-loaded TO901317-treated J744 (*top*) or MP (*bottom*) macrophages were incubated without or with 10 μg/ml of apoA-I for 3 h, and treated with 10 ng/ml of LPS for 3 h. TNF-α and IL-6 mRNA levels were measured and expressed as percentage of no apoA-I values. Results are mean ± S.D. of triplicate values from two separate experiments representative of four similar experiments. *, $p < 0.003$ compared with controls. *D*, cholesterol-loaded peritoneal macrophages were pre-treated with the indicated concentrations of STAT3 phosphorylation (*P-STAT3*) inhibitor for 2 h and then treated with or without 10 μg/ml apoA1 for 1 h. Cell proteins were immunoblotted with P-STAT3 antibody and then re-probed with total STAT3 antibody. *E*, cholesterol-loaded MP macrophages were pretreated for 2 h without (*Ctrl*) or with 10 μM P-STAT3 inhibitor (*Inhib*), incubated without or with inhibitor or 10 μg/ml of apoA-I for 3 h, and treated with 10 ng/ml LPS for 3 h. TNF-α and IL-6 mRNA levels were measured and expressed as percentage of no apoA1 values. Results are mean ± S.D. of quadruplicate values from two separate experiments. *, $p < 0.008$ compared with controls.

with a scrambled siRNA (Fig. 4*B*). The ability of apoA-I to reduce LPS-stimulated expression of TNF-α (or IL-6) was partially but significantly ($p < 0.03$) reversed by knocking down STAT3 (Fig. 4*C*). In this experiment, apoA-I decreased TNF-α and IL-6 mRNA levels by 46 and 56% in control cells, respectively, and this was reduced to 25 and 26% in STAT3 siRNA-transfected cells. These differences are comparable with the degree of silencing of STAT3 by the siRNA. These findings support the conclusion that apoA-I suppresses inflammation in J774 macrophages through the JAK2/STAT3 pathway.

Because J774 macrophages were relatively resistant to silencing STAT3 with a siRNA, we used another macrophage cell line to confirm the involvement of STAT3 in the anti-inflammatory effects of apoA-I. With isolated mouse peritoneal macrophages, transfection with the same STAT3 siRNA reduced STAT3 protein levels by ~80% (Fig. 4*B*). Also in contrast to J774 macrophages, we were able to induce ABCA1 to high levels by loading them with acetylated LDL-derived cholesterol without the need to treat them with an LXR agonist (data not shown). As with J774 macrophages, 3-h incubations of cholesterol-loaded peritoneal macrophages with apoA-I inhibited LPS-induced expression of both TNF-α and IL-6 (Fig. 4*C*). Transfecting cells with the STAT3 siRNA almost completely reversed the inhibitory effects of apoA-I on LPS-induced expression of TNF-α and IL-6 (Fig. 4*C*). Thus, our results show that apoA-I interactions with two different types of ABCA1-expressing macrophages inhibits inflammatory cytokine production by a STAT3-dependent process.

To examine if activation of STAT3 is required for the anti-inflammatory effects of apoA-I/ABCA1 interactions, we treated macrophages with the cell-permeable STAT3 inhibitor peptide (18). This inhibitor markedly reduced STAT3 phosphorylation in choles-

terol-loaded peritoneal macrophages without affecting total STAT3 protein levels (Fig. 4*D*). Whereas apoA-I reduced subsequent LPS-stimulated production of TNF-α and IL-6 by ~60% in these cells, these apoA-I effects were reduced to less than 30% in the presence of the STAT3 phosphorylation inhibitor (Fig. 4*E*). These findings support the idea that STAT3 activation is involved in the anti-inflammatory effects of apoA-I.

We used cholesterol-loaded peritoneal macrophages from mice lacking macrophage STAT3 to further confirm a role of STAT3 in the apoA-I-mediated suppression of cytokine production. As an additional control, we compared the effects of apoA-I with those of IL-10, a cytokine receptor system that suppresses macrophage inflammation through STAT3 (9). Because general knock-out of STAT3 is embryonic lethal (19), we generated mice with STAT3 silenced specifically in myeloid cells by breeding $STAT3^{fl/fl}$ mice with a mouse Lys-M-Cre line, which is under control of the macrophage/granulocyte-specific lysozyme-M promoter. Immunoblots of peritoneal and bone marrow macrophages from these mice showed a complete absence of STAT3 as compared with cells from wild-type animals ($STAT3^{wt/wt}$/Lys-M-Cre) or the whole population of white blood cells from $STAT3^{fl/fl}$/Lys-M-Cre mice (Fig. 5*A*).

Knocking out STAT3 markedly reduced the ability of apoA-I to suppress cytokine production from cholesterol-loaded peritoneal macrophages (Fig. 5*B*), supporting the idea that STAT3 plays a critical role in the anti-inflammatory effects of macrophage/apoA-I interactions. The ability of IL-10 to inhibit LPS-induced cytokine production was completely absent in cells lacking STAT3, consistent with IL-10 acting exclusively through STAT3. These findings show that apoA-I mimics IL-10 in suppressing inflammatory cytokine production by LPS-treated macrophages.

We also confirmed a role of ABCA1 in the apoA-I-mediated suppression of cytokine production by measuring the effects of apoA-I on cholesterol-loaded peritoneal macrophages from $ABCA1^{-/-}$ mice. The ability of apoA-I to suppress production of TNF-α and IL-6 was absent in macrophages lacking ABCA1 (Fig. 5*C*), indicating a complete requirement for this transporter for the anti-inflammatory effects of apoA-I.

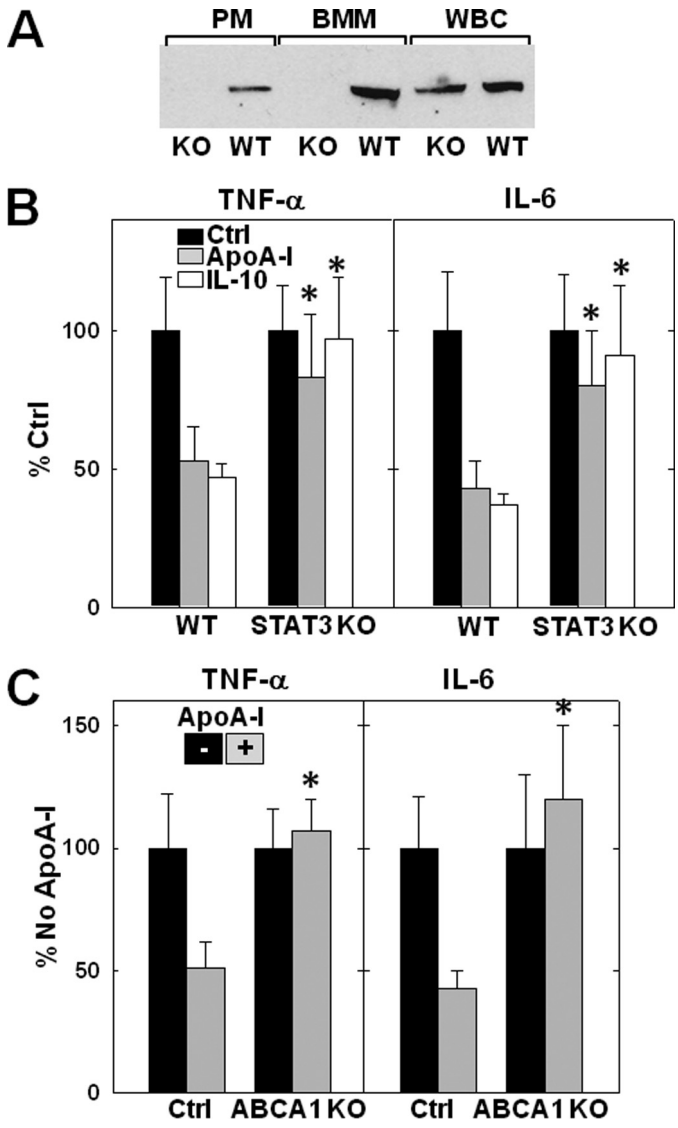

FIGURE 5. **Loss of anti-inflammatory activity of apoA-I in macrophages from myeloid-specific STAT3 and ABCA1 knock-out mice.** *A*, STAT3 immunoblots of peritoneal macrophages (*PM*), bone marrow macrophages (*BMM*), or white blood cells (*WBC*) from $STAT3^{fl/fl}$/LYS-M-CRE (*KO*) or $STAT3^{wt/wt}$/LYS-M-CRE mice (*WT*). *B*, cholesterol-loaded macrophages from WT or myloid-specific STAT3 KO mice were pretreated for 3 h with apoA-I or 10 ng/ml of IL-10 and incubated for 3 h with LPS, and cytokine mRNA levels were measured. Results are the mean ± S.D. from six values representing two experiments. *, $p < 0.003$ compared with WT. *C*, cholesterol-loaded macrophages from $ABCA1^{-/-}$ mice were pretreated for 3 h with apoA-I and incubated for 3 h with LPS, and cytokine mRNA levels were measured. Results are the mean ± S.D. from six values representing two experiments. *, $p < 0.001$ compared with controls.

DISCUSSION

We showed previously that the interaction of apoA-I and its mimetic peptides with ABCA1-expressing cells rapidly activates JAK2, and this enhances the direct interaction of apoA-I with ABCA1 required for lipid removal from the cells (6, 7). Here we show that this interaction also activates the transcription factor STAT3, and this is independent of the lipid transport activity of ABCA1. STAT3 has an anti-inflammatory function in macrophages (9), a major ABCA1-expressing cell type, and we found that the interaction of apoA-I with cholesterol-loaded macrophages suppressed LPS-induced production of inflammatory cytokines. Silencing STAT3 or ABCA1 in macrophages reduced the ability of apoA-I to inhibit cytokine production, indicating a critical role of these two proteins in the anti-inflammatory effects of apoA-I. These findings show that the interaction of apoA-I with ABCA1-expressing macrophages activates a JAK2/STAT3 pathway that has anti-inflammatory activity.

The two intracellular loops of ABCA1 (NBDs) each contain a tetrameric amino acid motif (Y*XX*Q) previously shown to create STAT3 docking sites in cytokine receptors (15). Mutating these sites completely abolished the ability of apoA-I to activate and bind STAT3 without affecting the cholesterol export function of ABCA1, indicating that these sites are responsible for the apoA-I-mediated STAT3 activation. Remarkably, the site in NBD2 lies in a pentamer (GYPCQ) that has the identical sequence as the prototypic STAT3 docking site originally described for the gp130 subunit of cytokine receptors (15). Mutating this site had the largest effect of preventing apoA-I from activating STAT3.

Whereas the JAK2/STAT3 pathway is oncogenic and pro-inflammatory in many cell types (20), it has an anti-inflammatory function in macrophages. Constitutive expression of active STAT3 in cultured macrophages nearly abolishes LPS-induced inflammatory cytokine production (9). Selective silencing of STAT3 in mouse macrophages and neutrophils increases susceptibility to endotoxic shock and promotes chronic entercolitis (21–23). The anti-inflammatory cytokine IL-10 suppresses production of inflammatory cytokines in macrophages through STAT3, and IL-10 knock-out mice have chronic inflammation and increased atherosclerosis (24, 25).

Here we found that pretreating ABCA1-expressing macrophages with apoA-I suppressed subsequent LPS-induced production of the inflammatory cytokines TNF-α, IL-1β, and IL-6. ApoA-I therefore primes ABCA1-expressing macrophages to resist LPS-induced inflammation. This effect of apoA-I was reduced by knocking down STAT3 with an siRNA, silencing STAT3 in myeloid-specific STAT3 knock-out mice, or inhibiting STAT3 phosphorylation, indicating a role for STAT3 in the anti-inflammatory effects of apoA-I. A role for ABCA1 was confirmed by results showing that apoA-I lost its anti-inflammatory activity in macrophages from ABCA1$^{-/-}$ mice.

Silencing STAT3 in macrophages did not completely reverse the ability of apoA-I to suppress LPS-induced cytokine production, indicating that some of the anti-inflammatory effects of apoA-I are independent of STAT3. Based on studies with macrophages lacking STAT3, it was estimated that 65% of the effects of apoA-I on LPS-induced inflammatory cytokine production could be attributed to STAT3. It has been shown that the cholesterol export activity of apoA-I can also inhibit macrophage inflammatory cytokine production by disrupting sterol-rich membrane rafts (26–30). It is therefore likely that apoA-I/ABCA1 interactions can suppress macrophage inflammation by multiple mechanisms. The availability of mutant forms of ABCA1 that lack the ability to activate STAT3 but retain lipid export activity will allow us to examine the contribution of these different mechanisms to the overall anti-inflammatory effects of apoA-I.

There is controversy about whether or not selective deletion of macrophage ABCA1 modulates atherosclerosis. Transplantation of *Abca1*-deficient bone marrow into mice significantly reduced atherosclerosis (31, 32), but myeloid-specific silencing of ABCA1 in whole animals had no effect on atherosclerosis (33). One explanation for these discrepancies might be related to the macrophage-like cell types that lack ABCA1 in the atherosclerotic lesions. Of particular interest is the fact that the myeloid-specific ablation of ABCA1 had little effect on the dendritic cell ABCA1, a cell type that may play an important role in atherogenesis (34). Loading macrophages with cholesterol induces a dendritic cell phenotype (35), and STAT3 negatively regulates dendritic cell function (36). It is also not known if overexpression of ABCA1 in macrophages could have an anti-inflammatory effect.

Taken together, the current and previous studies (5–7) show that interaction of apoA-I with ABCA1-expressing cells rapidly activates JAK2, which in turn activates two independent pathways: lipid export from cells and STAT3-mediated transcription (Fig. 6). The observation that ABCA1 contains active

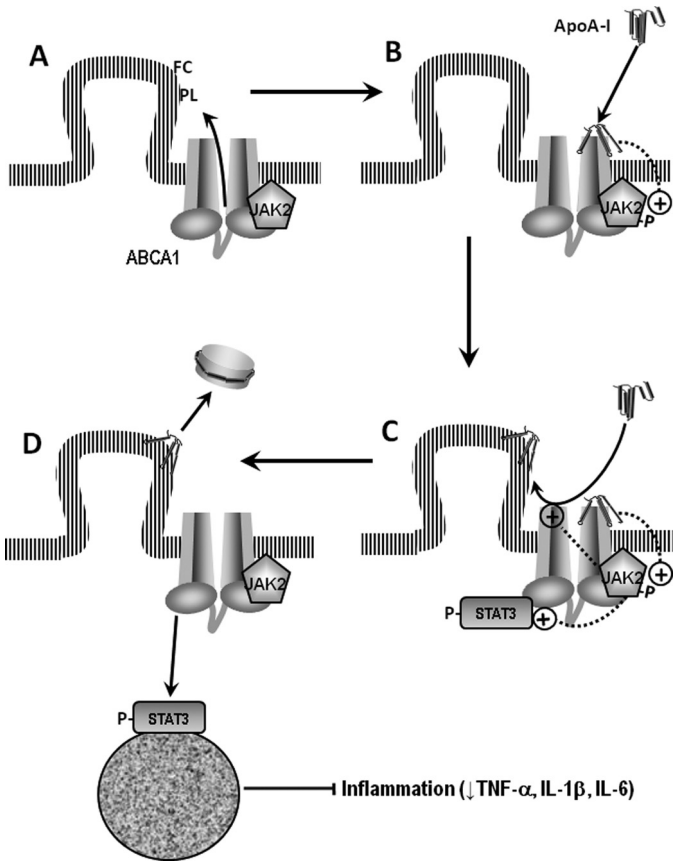

FIGURE 6. **A model for the ABCA1/JAK2-dependent branch pathway for activation of cholesterol export and STAT3-mediated suppression of inflammatory cytokine production by macrophages.** *A*, ABCA1 intrinsically forms cell-surface lipid domains enriched with phospholipids (*PL*) and free cholesterol (*FC*). *B*, apoA-I binds to ABCA1, stimulating autophosphorylation (activation) of associated JAK2. *C*, the activated JAK2 both enhances the interaction of apoA-I with ABCA1 required for lipid interactions and creates STAT3 docking sites that promote JAK2-mediated phosphorylation of STAT3. *D*, the lipid-bound apoA-I solubilizes the ABCA1-formed lipid domains to generate nascent HDL particles, and the phosphorylated STAT3 is translocated to the nucleus where it regulates transcription events that suppress LPS-mediated production of inflammatory cytokines.

STAT3 docking sites that allow it to function as a signaling receptor is a unique property among cell membrane transporters. One novel feature of ABCA1 compared with other eucharyotic transporters is that its export function requires direct molecular interactions between the transporter and the acceptor of the transported cargo. This creates the opportunity for ligand/receptor-like interactions to regulate both transport activity and signaling pathways.

Studies of human HDL deficiencies and mouse models have shown that loss-of-function mutations in ABCA1 increase cardiovascular disease (4, 31). This has been attributed to an impaired removal of excess cholesterol from macrophages by HDL apolipoproteins (37), which would lead to accumulation of sterol-laden macrophages in the artery wall. Mice lacking ABCA1 in all tissues or selectively in macrophages have an enhanced inflammatory response to LPS (26, 38, 39), implying that macrophage ABCA1 has an anti-inflammatory function *in vivo* and that this may contribute to its cardioprotective effects. Studies of isolated macrophages from these mice suggest that this may be secondary to the inability of ABCA1 to export lipids

from cells, which modifies plasma membrane lipid rafts and enhances LPS signaling (27). Our studies show that ABCA1 can also directly suppress LPS-induced inflammatory cytokine production by macrophages through a STAT3 pathway that is independent of cholesterol export.

This novel dual function of ABCA1 may have evolved as part of the innate immune system, particularly as a component of the resolution phase of inflammation (40). Activated macrophages at sites of inflammation release inflammatory cytokines and phagocytose apoptotic cells, which contain cholesterol-rich membranes. An unchecked accumulation of cholesterol in macrophages can be cytotoxic and evoke exaggerated inflammatory responses (26, 41), thus promoting sustained tissue damage. The induction of ABCA1 by the accumulating cholesterol would divert the cholesterol from cells into the HDL pathway and prevent cytotoxicity and excessive inflammation. In parallel, the induced ABCA1 would resolve the inflammatory response through activation of JAK2/STAT3. The same process is likely to occur in atherosclerotic lesions, except that the major source of macrophage-engulfed cholesterol would be sterol-rich lipoproteins trapped in the artery wall. Thus, ABCA1 may be a direct link between the cardioprotective effects of cholesterol export and anti-inflammation. These findings also raise the interesting possibility that ABCA1 might have a protective role in other inflammatory disorders characterized by the local accumulation of macrophages.

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