Both STAT3 activation and cholesterol efflux contribute to the anti-inflammatory effect of apoA-I/ABCA1 interaction in macrophages

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Abstract ABCA1 exports excess cholesterol from cells to apoA-I and is essential for HDL synthesis. Genetic studies have shown that ABCA1 protects against cardiovascular disease. We have previously shown that the interaction of apoA-I with ABCA1 activates signaling molecule Janus kinase 2 (JAK2), which optimizes the cholesterol efflux activity of ABCA1. ABCA1-mediated activation of JAK2 also activates signal transducer and activator of transcription 3 (STAT3), which significantly attenuates proinflammatory cytokine expression in macrophages. To determine the mechanisms of the anti-inflammatory effects of apoA-I/ABCA1 interaction, we identified two special ABCA1 mutants, one with normal STAT3-activating capacity but lacking cholesterol efflux ability and the other with normal cholesterol efflux ability but lacking STAT3-activating capacity. We showed that activation of STAT3 by the interaction of apoA-I/ABCA1 without cholesterol efflux could significantly decrease proinflammatory cytokine expression in macrophages. Mechanistic studies showed that the anti-inflammatory effect of the apoA-I/ABCA1/STAT3 pathway is suppressor of cytokine signaling 3 dependent. Moreover, we showed that apoA-I/ABCA1-mediated cholesterol efflux without STAT3 activation can also reduce proinflammatory cytokine expression in macrophages. These findings suggest that the interaction of apoA-I/ABCA1 activates cholesterol efflux and STAT3 branch pathways to synergistically suppress inflammation in macrophages. — Chongren, T., B. A. Houston, C. Storey, and R. C. LeBoeuf. Both STAT3 activation and cholesterol efflux contribute to the anti-inflammatory effect of apoA-I/ABCA1 interaction in macrophages. J. Lipid Res. 2016. 57: 848–857.

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Population studies have shown an inverse relationship between circulating levels of HDL and risk for cardiovascular disease (1–3). The atheroprotective effects of HDL have traditionally been attributed to its role in reverse cholesterol transport, implying that factors associated with HDL metabolism and its reverse cholesterol transport ability are cardioprotective (4, 5). One of these factors is ABCA1, which plays a crucial role in mediating cholesterol efflux from peripheral cells, including arterial wall macrophages, to lipid-poor apoA-I or pre-B HDL particles (6–9). Loss-of-function mutations in human ABCA1 increase the prevalence and severity of atherosclerosis (6, 7, 10, 11), although one study indicates that heterozygosity for loss-of-function mutations in ABCA1 is not associated with an increased risk of ischemic heart disease (12). Humans with dysfunctional ABCA1 and familial HDL deficiencies tend to have chronic low-grade inflammation (13, 14). ABCA1 mutation carriers also have increased systemic and plaque inflammation (15). Overexpression of human ABCA1 in some transgenic atherogenic mouse models protects against atherosclerosis (16), and ablation of the ABCA1 gene selectively in bone-marrow-derived cells increases atherosclerotic lesions in mice (17, 18). ABCA1−/− mice crossed to atherogenic LDLR−/− mice have been shown to have higher levels of inflammatory cytokines in the blood and peritoneal fluid compared with mice expressing ABCA1, especially when acutely inflamed (19). Cultured peritoneal macrophages from ABCA1−/− mice produce more inflammatory cytokines than cells from WT mice (20).

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 (21, 22). We reported previously that the interaction of apoA-I or its synthetic mimetic peptides with ABCA1 activates Janus kinase 2 (JAK2), which in turn enhances the apo-A-I binding activity of ABCA1 responsible for lipid removal (22, 23). We also showed that the interaction of apoA-I or its mimetic peptides with ABCA1 activates the transcription factor signal transducer and activator of transcription 3 (STAT3) (24). The STAT3 pathway is well known to have an anti-inflammatory function in macrophages (25–27). In fact, constitutive activation of STAT3 is sufficient to block most of the activated macrophage production of inflammatory cytokines (28). We have shown that the interaction of apoA-I or its mimetic peptides with ABCA1-expressing macrophages activates STAT3 and markedly suppresses the expression of proinflammatory cytokines (24). However, STAT3 activation is not required for ABCA1-mediated cholesterol efflux (24). For the first time, these findings link apoA-I, ABCA1, and STAT3 together, supporting the idea that ABCA1 can directly function as an anti-inflammatory receptor through STAT3. Therefore, we hypothesize that, in addition to its cholesterol export activity that may have anti-inflammatory effects, ABCA1 also functions as an anti-inflammatory signaling receptor in macrophages through the activation of STAT3 by interacting with apolipoproteins independent of cholesterol export.

To test this hypothesis, we developed two novel ABCA1 mutants, one with normal STAT3-activating capacity but lacking cholesterol efflux ability and the other with normal cholesterol efflux ability but lacking STAT3-activating capacity. We showed that activation of STAT3 by the interaction of apoA-I/ABCA1 without cholesterol efflux significantly decreased proinflammatory cytokine expression in macrophages. Mechanistic studies showed that the anti-inflammatory effect of the apoA-I/ABCA1/STAT3 pathway is suppressor of cytokine signaling 3 (SOCS3) dependent. Moreover, we show that apoA-I/ABCA1-mediated cholesterol efflux without STAT3 activation can also reduce inflammatory cytokine expression in macrophages. These findings demonstrate that apoA-I/ABCA1 interaction-mediated STAT3 activation and cholesterol efflux act synergistically to suppress inflammation in macrophages.

MATERIALS AND METHODS

Antibodies and reagents

STAT3, phospho-STAT3, and phospho-JAK2 antibodies were purchased from Cell Signaling Technology (MA); STAT1, phospho-STAT1, JAK2, and SOCS3 antibodies were purchased from Santa Cruz Biotechnology (TX). ABCA1 antibody was purchased from Novus Biological (CO). ApoA-I was purified from HDL as described previously (29).

Mice

ABCA1<sup>-/-</sup>/DBA mice were a gift from Robert Aiello, Pfizer-Wyeth, STAT3<sup>fl</sup>/<sup>fl</sup>/Lys-M-Cre/C57BL/6 mice were a gift from Shizuo Akira, Osaka University. 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<sup>flox</sup>/<sup>flox</sup>/Lys-M-Cre mice were generated by interbreeding STAT3<sup>wt</sup>/<sup>wt</sup> with Lys-M-Cre mice (24). All mouse experimental procedures were undertaken with approval from the Institutional Animal Care and Use Committee of the University of Washington.

Plasmid constructs and cell transfection

WT and mutant ABCA1 were generated using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, CA) as described previously (30). All the constructs and mutations were verified by DNA sequencing, to confirm both the introduction of the desired changes and the absence of unintended PCR mutations. Baby hamster kidney (BHK) cells or thioglycollate-elicited peritoneal macrophages were transiently transfected with WT-ABCA1 or mutants using Lipofectamine 2000 (Invitrogen Corp., San Diego, CA). Twenty-four hours after transfection, cells were sorted using fluorescence-activated cell sorting (FACS), and ABCA1-expressing cells were selected and recultured in 12- to 24-well plates in DMEM with 10% fetal bovine serum until experiments.

Cellular lipid efflux and apoA-I cell binding

Cells were labeled with 1 μCi/ml of [3H]cholesterol (PerkinElmer Life Sciences) for cholesterol efflux or [3H]choline (PerkinElmer Life Sciences) for phospholipid efflux in DMEM/BSA medium overnight. Washed cells were then equilibrated for 18 h, followed by incubating with DMEM/BSA minus or plus 10 μg/ml apoA-I for the indicated time, and medium and cells were assayed for [3H] counts (22). ApoA-I-mediated lipid efflux was calculated as the percent total [3H] released into medium after subtraction of values obtained in the absence of apoA-I. For apoA-I binding to ABCA1 studies, cells were incubated with 5 μg/ml [125<sup>I</sup>]apoA-I for 2 h, treated for 30 min at room temperature with PBS containing 1 mg/ml dithiobis succinimidyl propionate (cross-linking agent), and washed twice with cold PBS containing 20 mM glycine (30). ABCA1/apoA-I complexes were isolated from detergent extracts by immunoprecipitation, and [125<sup>I</sup>]apoA-I was visualized by phosphorimaging and quantified using Opti-Quant computer software (Packard Instruments). For apoA-I cell association, cells were incubated with 0.2 μg/ml [125<sup>I</sup>]apoA-I in 0.2% BSA and DMEM for 1 h at 37°C. After being washed three times with fresh media, cells were lysed with 0.1% SDS and 0.1 N NaOH lysis buffer, and radioactivity was determined by a γ counter.

siRNA

Transfected and sorted thioglycollate-elicited mouse peritoneal macrophages were plated in 35 mm or 24-well plates and maintained in DMEM with 10% (v/v) heat-inactivated fetal bovine serum, penicillin, and streptomycin. SOCS3 siRNA and silencer negative control siRNA (Santa Cruz Biotechnology) and transfection were performed with Lipofectamine (Life Technologies, Carlsbad, CA) according to the manufacturers’ instructions. One day after transfection, medium was changed, and cells were loaded overnight with 50 μg/ml of acetylated LDL in DMEM. Cells were then treated with or without 10 μg/ml of apoA-I for 3 h, washed twice, and then treated with without 10 ng/ml of lipopolysaccharide (LPS) for 3 h. Cells were collected for RT-PCR analyses.

Immunobots 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 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 cell surface ABCA1 analysis, cells were first biotinylated with 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce Chemical Co., Rockford, IL) at 4°C for 30 min. Then cells were lysed with RIPA buffer at 4°C. After centrifugation, the supernatant of cell lysates was incubated with anti-ABCA1 agarose beads overnight at 4°C. Following centrifugation and washing, the collected agarose beads were subjected to SDS-PAGE sample buffer with 100 mM 2-mercaptoethanol. Cell surface ABCA1 was detected by Western blot using streptavidin-horseradish peroxidase. Band intensity was quantified from the autoradiograph scans using OptiQuant computer software (Packard Instruments).

**Real-time quantitative PCR**

Total RNA was extracted from cultured cells using a commercially available RNA extraction kit according to the manufacturer’s protocol (Agilent Technologies, Santa Clara, CA). After spectrophotometric quantification, 2 μg of RNA was reverse-transcribed, and cDNA thus obtained was analyzed by real-time quantitative PCR. Primers specific for individual genes were purchased from Applied Biosystems (Assay-on-Demand; Life Technologies). GAPDH and L32 were used as the control housekeeping genes. Relative amounts of the target gene were calculated using the ΔΔCt formula.

**Measurement of plasma membrane cholesterol and lipid rafts**

Cellular membranes were isolated from cells as described previously (31). Membrane preparations were resuspended in 100 μl of ethanolic potassium hydroxide (1 M), and cholesterol-d7 was added as the internal standard. After saponification, the lipid fraction was extracted from the membrane preparations with hexane and dried under nitrogen gas. Total cholesterol levels were determined after derivatization using GC/MS, as described previously (31, 32).

Lipid rafts in plasma membranes of cells were quantified as described previously using Alexa Fluor 594 conjugated cholera toxin subunit (CTB) (31). Briefly, cultured macrophages were incubated with 1 μg/ml of Alexa Fluor 594 conjugated CTB for 15 min at 4°C. After washing twice with cold PBS, cells were fixed in 4% paraformaldehyde for 20 min at 4°C. CTB staining of fixed cells was analyzed by Western blot (FACS Canto, Becton-Dickinson) as in 4% paraformaldehyde for 20 min at 4°C. CTB staining of fixed cells was analyzed by Western blot (FACS Canto, Becton-Dickinson) as described previously (31, 32).

**Statistical analyses**

Data are presented as mean and standard deviation and were analyzed using the GraphPad Prism 5 program (GraphPad Software Inc., La Jolla, CA). The Student’s t-test was used to detect differences within groups when applicable. One-way ANOVA was used to compare differences among all groups, and Bonferroni post hoc testing was used to detect differences among mean values of the groups. A P value <0.05 was considered as statistically significant.

**RESULTS**

**Identify and characterize ABCA1 mutants with unique cholesterol efflux and STAT3-activating capacity**

We showed previously that knockout of STAT3 or mutation of the STAT3 docking sites of ABCA1 markedly reduces the ability of apoA-I to suppress cytokine production from cholesterol-loaded macrophages (24). These data demonstrate that STAT3 plays a critical role in the anti-inflammatory effects of apoA-I/ABCA1 interaction in macrophages. In addition, several studies suggested that altering the cholesterol content of macrophages modulates macrophage inflammatory responses (19, 20, 33, 34). It is therefore possible that the apoA-I/ABCA1 interaction suppresses inflammatory cytokine production by both activating STAT3 and removing excess cholesterol from cells. We have shown that ABCA1-mediated STAT3 activation and cholesterol efflux are two separable events. The key question is whether STAT3 activation by apoA-I/ABCA1 interaction without cholesterol efflux is enough to mediate the anti-inflammatory effects of apoA-I/ABCA1 interaction.

We showed previously that the STAT3 docking site mutant Y924F-Y1990F located on ABCA1 retains normal cholesterol export activity but lacks STAT3-activating capacity. Here, we generated another ABCA1 mutant that retains normal STAT3-activating capacity but has impaired cholesterol export activity. PCR mutagenesis was used to create an ABCA1 mutant library, and mutants were screened for the ability to activate STAT3 and to efflux cholesterol by apoA-I/ABCA1 interaction. For better visualization of ABCA1 expression and sorting, a green fluorescent protein tag was added to the C terminal of ABCA1, and this does not affect ABCA1 function (35–37).

Mutants and WT-ABCA1 were transiently transfected into BHK cells, and the level of ABCA1 expression, the ability to mediate cholesterol and phospholipid efflux, and the ability to activate STAT3 (promote STAT3 phosphorylation) were measured. When expressed at similar levels as WT-ABCA1 (Fig. 1A), the STAT3 docking site mutant Y924F-Y1990F showed normal cholesterol (Fig. 1B) and phospholipid efflux (Fig. 1C) activity but lacked apoA-I-induced STAT3-activating capacity (STAT3 phosphorylation) (Fig. 1D). Significantly, the W590R mutant showed similar apoA-I-induced STAT3-activating capacity as WT-ABCA1 (Fig. 1D) but was unable to mediate the efflux of cholesterol and phospholipid (Fig. 1B, C). Moreover, another extracellular loop mutant, Q597R [causative for Tangier disease in humans (30)], showed no apoA-I-induced STAT3 activation or cholesterol and phospholipid efflux capacity (Fig. 1).

We and others have previously shown that the expression of ABCA1 at the cell surface and the ability to bind to apoA-I are required for ABCA1-mediated cholesterol efflux (30, 35). To further characterize our mutants and determine why W590R and Q597R lose their ability to efflux cholesterol, we visualized the location of their expression using confocal microscopy and measured cell surface expression levels using immunoblotting. As shown by confocal microscopy, WT and Y924F-Y1990F, W590R, and Q597R mutants were all expressed and transported to the cell surface (Fig. 2A). FACS analyses (Fig. 2B) and biotin cross-linking immunoblotting analyses indicated that cell surface levels of Y924F-Y1990F, W590R, and Q597R were comparable to WT (Fig. 2C). We then determined the binding ability of these mutants to apoA-I using
cross-linking. Interestingly, W590R was able to bind to apoA-I as well as WT, but the binding ability of Q597R to apoA-I was significantly impaired (Fig. 2D). Finally, we studied how the mutant proteins associated with apoA-I and found that apoA-I cell association was not affected in cells expressing W590R or Y924F-Y1990F. However, apoA-I cell association was significantly reduced in cells expressing the Q597R (Fig. 2E). These data suggest that apoA-I binding is necessary for STAT3 activation and that apoA-I binding is required but not sufficient for ABCA1-mediated cholesterol efflux.

STAT3 activation by apoA-I in the absence of cholesterol efflux by apoA-I/ABCA1 interaction significantly decreased proinflammatory cytokine expression in macrophages

Having confirmed that W590R is able to activate STAT3 but is not able to mediate cholesterol efflux, we next tested if this mutant would be able to mediate the anti-inflammatory effect of apoA-I/ABCA1 interaction in macrophages. We transfected WT, Y924F-Y1990F, W590R, and Q597R into peritoneal macrophages isolated from ABCA1−/− mice. It is essential to have the WT and mutants expressed in most of the transfected cells and at similar levels. FACS analyses revealed that ~65% of the in vitro transfected peritoneal macrophages were green fluorescent protein positive. However, after FACS sorting, the proportions were close to 98% for WT and mutant cells (data not shown). Consistent with efflux data in transfected BHK cells, apoA-I-mediated cholesterol efflux was similar in macrophages transfected with WT and Y924F-Y1990F, whereas apoA-I mediated cholesterol efflux

Fig. 2. ABCA1 mutant W590R does not affect cell surface expression or apoA-I association. WT and ABCA1 mutants Y924F-Y1990F, W590R, and Q597R were transiently transfected into BHK cells and sorted by FACS. Sorted cells were recultured. A: Cell surface expression was visualized using confocal microscope. B: ABCA1 expression was analyzed by FACS. C: Cell surface (biotinylated) ABCA1 expression was detected by immunoblots. D: ApoA-I binding to ABCA1 was detected by cross-linking. E: ApoA-I cell surface association (mean ± SD from four experiments). * P < 0.05 compared with WT.
were measured by RT-PCR. As shown in Fig. 4, apoA-I was able to significantly decrease the expression of TNF-α, IL-1β, and IL-6 in cells transfected with W590R, although not to the same extent as with WT-ABCA1. ApoA-I also slightly but not statistically significantly decreased the expression of TNF-α, IL-1β, and IL-6 in cells transfected with Y924F-Y1990F, and there is no effect in cells transfected with Q597R. These data indicate that activating STAT3 by apoA-I/ABCA1 interactions without cholesterol efflux can significantly inhibit proinflammatory cytokine expression in macrophages.

**ApoA-I/ABCA1/STAT3 pathway attenuated proinflammatory cytokine expression through SOCS3**

In a few instances, SOCS3 has been suggested to play a role in mediating the anti-inflammatory effects of STAT3 (38, 39). To determine the possible mechanisms by which apoA-I/ABCA1/STAT3 inhibits inflammation, we investigated whether there was a corresponding regulation of SOCS3 by apoA-I/ABCA1 interaction and whether SOCS3 is required for the anti-inflammatory effects of the apoA-I/ABCA1/STAT3 pathway. As shown in Fig. 5A–C, apoA-I significantly increased SOCS3 expression in peritoneal macrophages derived from WT mice (control) but not in peritoneal macrophages derived from ABCA1−/− mice. ApoA-I also increased SOCS3 expression in peritoneal macrophages transfected with WT and W590R but not with Q597R or Y924F-Y1990F (Fig. 5D–F), implying that apoA-I increases SOCS3 expression in an ABCA1-dependent and a STAT3-dependent manner. We then tested if SOCS3 is important for the anti-inflammatory effects of apoA-I/ABCA1/STAT3 pathway by silencing SOCS3 using SOCS3-specific siRNA. SOCS3 silencing in macrophages was confirmed by demonstrating significant reduction of its expression level by RT-PCR and immunoblotting (Fig. 6A–C). When SOCS3 was silenced, the effect of apoA-I on inhibiting TNFα, IL-1β, and IL-6 gene expression induced by LPS was reversed (Fig. 6D–F). These data indicate that there is a requirement for SOCS3 expression for the anti-inflammatory effects of apoA-I/ABCA1/STAT3 pathway. Because IL-10 has been shown to play an anti-inflammatory role in macrophages, we checked whether apoA-I/ABCA1 interaction increases the expression of IL-10. We found that IL-10 expression was not changed by the apoA-I/ABCA1 interaction (data not shown), suggesting that the anti-inflammatory effects of apoA-I/ABCA1 interaction are not through IL-10.

**ApoA-I/ABCA1 interaction-mediated cholesterol efflux without STAT3 activation reduced membrane cholesterol levels and lipid raft formation and inhibited proinflammatory cytokine production in macrophages**

Cholesterol is an essential component of lipid rafts (40). Depleting cholesterol in the plasma membrane by HDL and other reagents disrupts lipid rafts and blocks the assembly of proteins, resulting in inhibition of inflammatory signal transduction (40, 41). We have previously shown that apoA-I reduces proinflammatory cytokine expression...
Fig. 4. Activating STAT3 by apoA-I/ABCA1 interactions without cholesterol efflux inhibits proinflammatory cytokine production in macrophages. WT and ABCA1 mutants Y924F-Y1990F, W590R, and Q597R were transiently transfected into cultured mouse peritoneal macrophages derived from ABCA1−/− mice. The transfected macrophages were sorted by FACS, recultured, and loaded for 24 h with 50 μg/ml of acetyl-LDL-derived cholesterol, 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 (A), IL-1β (B), and IL-6 (C) mRNA levels were measured by RT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels. Results are mean ± SD of four separate experiments. * P < 0.05 compared with no apoA-I; † P < 0.05 compared with WT.

Fig. 5. ApoA-I/ABCA1 interaction increases SOCS3 expression in an ABCA1/STAT3-dependent mechanism. A–C: Mouse peritoneal macrophages derived from WT (control), ABCA1−/−, or STAT3−/− mice were cultured and loaded for 24 h with 50 μg/ml of acetyl-LDL-derived cholesterol, incubated without or with 10 μg/ml of apoA-I for 3 h, washed and cultured for another 3 h. SOCS3 mRNA and protein levels were measured by RT-PCR and immunoblot, respectively. A: SOCS3 mRNA expression. B: Representative of immunoblots. C: Quantification of immunoblots. D–F: Transfected and sorted mouse peritoneal macrophages derived from ABCA1−/− were cultured and loaded for 24 h with 50 μg/ml of acetyl-LDL-derived cholesterol, incubated without or with 10 μg/ml of apoA-I for 3 h, washed, and cultured for another 3 h. SOCS3 mRNA and protein levels were measured by RT-PCR and immunoblot, respectively. D: SOCS3 mRNA expression. E: Representative of immunoblots. F: Quantification of immunoblots. Results are mean ± SD of four separate experiments. * P < 0.01 compared with no apoA-I. Aus, arbitrary units.
disturbance of lipid rafts, which in turn inhibits inflammatory cytokine expression.

**DISCUSSION**

We showed previously that the interaction of apoA-I or its mimetic peptides with ABCA1-expressing cells rapidly activates JAK2, which enhances the direct interaction of apoA-I with ABCA1 (22, 23). We also showed that the interaction of apoA-I or its mimetic peptides with ABCA1 activates STAT3 and markedly suppresses the expression of proinflammatory cytokines in macrophages (24). However, STAT3 activation is not required for ABCA1-mediated cholesterol efflux (24). Several studies have suggested that altering cholesterol content of macrophages can affect macrophage inflammatory responses (19, 20, 33, 34). Using two novel ABCA1 mutants, one with normal STAT3-activating capacity but lacking cholesterol efflux ability and the other with normal cholesterol efflux ability but lacking STAT3-activating capacity, we found that activating STAT3 without cholesterol efflux by apoA-I/ABCA1 interaction significantly inhibited proinflammatory cytokine expression in macrophages, but not to the same extent as activating STAT3 with cholesterol efflux. We also found that apoA-I/ABCA1-interaction-mediated cholesterol efflux without STAT3 activation can also significantly reduce inflammatory cytokine expression in macrophages.
Mechanism of anti-inflammatory effect of apoA-I/ABCA1 interaction

When the efflux is significant enough to alter plasma membrane cholesterol content and lipid raft formation, these findings suggest that both STAT3 activation and cholesterol efflux contribute to the anti-inflammatory effects of apoA-I/ABCA1 interaction.

The JAK2/STAT3 pathway has an anti-inflammatory function in macrophages (28). Constitutive expression of active STAT3 in cultured macrophages nearly abolishes LPS-induced inflammatory cytokine production (28). Selective silencing of STAT3 in mouse macrophages and neutrophils increases susceptibility to endotoxic shock and promotes chronic enterocolitis (27, 43). The anti-inflammatory cytokine IL-10 suppresses the production of inflammatory cytokines in macrophages through STAT3, and IL-10 knockout mice have chronic inflammation and increased atherosclerosis (26, 44). Here we found that activating STAT3 without cholesterol efflux by the interaction of apoA-I/ABCA1 significantly inhibited proinflammatory cytokine expression in macrophages. It has been described that phosphorylated STAT3 is dimerized and induces nuclear transcription of SOCS3 gene (45). For example, SOCS3 has been reported as a key mediator in the inhibitory effects of IL-10 in macrophages stimulated with LPS (39, 46). In this work, we report that apoA-I/ABCA1 interaction can upregulate SOCS3 expression through the activation of STAT3. ApoA-I failed to induce SOCS expression in STAT3 or ABCA1 knockout macrophages, confirming that SOCS3 is induced by the apoA-I/ABCA1/STAT3 pathway in our model.

To further evaluate mechanisms involving the apoA-I/ABCA1/STAT3 anti-inflammatory effects, we investigated the consequence of the specific knockdown of SOCS3. We demonstrated that SOCS3 is essential for the anti-inflammatory effect of the apoA-I/ABCA1/STAT3 pathway, because its silencing prevented apoA-I/ABCA1/STAT3 inhibitory effects on proinflammatory expression.

Silencing STAT3 in macrophages did not completely reverse the ability of apoA-I to suppress LPS-induced cytokine production. The ABCA1 mutant with full STAT3-activating capacity but lacking cholesterol efflux ability is less effective in mediating the anti-inflammatory effect of apoA-I/ABCA1 than WT-ABCA1 (Figs. 4 and 7) (24). These findings indicate that some of the anti-inflammatory effects of apoA-I/ABCA1/STAT3 inhibitory effects on proinflammatory expression.

Based on our studies, it was estimated that 60% to 65% of the effects of apoA-I/ABCA1 interactions on LPS-induced inflammatory cytokine production could be attributed to STAT3. Indeed, we found that cholesterol efflux without STAT3 activation by the interaction of apoA-I/ABCA1 can significantly reduce inflammatory cytokine expression in macrophages, when the efflux is significant enough to alter plasma membrane cholesterol content and lipid raft formation. Although some controversy exists regarding the definition or even the existence of lipid rafts, proteins involved in cell signaling clearly cluster in certain domains of plasma membrane (17).
Accumulation of free cholesterol of the plasma membrane is associated with increased signaling via Toll-like receptor 4, which resides in these domains. Depleting cholesterol in the plasma membrane by HDL and other reagents disrupts those domains and blocks the assembly of proteins, resulting in inhibition of inflammatory signal transduction (31, 40). We found that when incubating apoA-I with ABCA1-expressing cells for up to 12 h significantly reduced the plasma membrane free cholesterol content and lipid raft formation. It is therefore likely that apoA-I/ABCA1 interaction suppresses macrophage inflammation by activating STAT3 and exporting cholesterol.

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 (47). Our results indicate that the interaction of apoA-I with ABCA1 activates the JAK2/STAT3 branch pathways to unload cholesterol and suppress inflammation. This novel dual function of ABCA1 may have evolved as part of defensive mechanisms of the innate immune system (48). Activated macrophages at sites of inflammation or atherosclerotic lesions accumulate cholesterol from apoptotic cells or cholesterol-rich lipoproteins. An unchecked accumulation of cholesterol in macrophages can be cytotoxic and can exaggerate inflammatory responses (41, 49). ABCA1 is one of the major proteins induced by cholesterol loading (50). Therefore, the induction of ABCA1 by the accumulation of cholesterol would divert the cholesterol from cells into the reverse cholesterol pathway and prevent cytotoxicity and excessive inflammation. In parallel, the induced ABCA1 would resolve the inflammatory response through activating STAT3. Thus, ABCA1 may be a direct link between the cardioprotective effects of cholesterol export and anti-inflammation.

In summary, the current and previous studies show that interaction of apoA-I with ABCA1 rapidly activates JAK2, which in turn activates two independent pathways, cholesterol export from cells and STAT3-mediated transcription, and both pathways contribute and act synergistically to suppress inflammation in macrophages. 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.25

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