Inflammation Impairs Reverse Cholesterol Transport In Vivo

Fiona C. McGillicuddy, PhD; Margarita de la Llera Moya, PhD; Christine C. Hinkle, MS; Michelle R. Joshi, BSc; Elise H. Chiquoine, BSc; Jeffrey T. Billheimer, PhD; George H. Rothblat, PhD; Muredach P. Reilly, MBBCh

Background—Inflammation is proposed to impair reverse cholesterol transport (RCT), a major atheroprotective function of high-density lipoprotein (HDL). The present study presents the first integrated functional evidence that inflammation retards numerous components of RCT.

Methods and Results—We used subacute endotoxemia in the rodent macrophage-to-feces RCT model to assess the effects of inflammation on RCT in vivo and performed proof of concept experimental endotoxemia studies in humans. Endotoxemia (3 mg/kg SC) reduced 3H-cholesterol movement from macrophage to plasma and 3H-cholesterol associated with HDL fractions. At 48 hours, bile and fecal counts were markedly reduced consistent with downregulation of hepatic expression of ABCG5, ABCG8, and ABCB11 biliary transporters. Low-dose lipopolysaccharide (0.3 mg/kg SC) also reduced bile and fecal counts, as well as expression of biliary transporters, but in the absence of effects on plasma or liver counts. In vitro, lipopolysaccharide impaired 3H-cholesterol efflux from human macrophages to apolipoprotein A-I and serum coincident with reduced expression of the cholesterol transporter ABCA1. During human (3 ng/kg; n=20) and murine endotoxemia (3 mg/kg SC), ex vivo macrophage cholesterol efflux to acute phase HDL was attenuated.

Conclusions—We provide the first in vivo evidence that inflammation impairs RCT at multiple steps in the RCT pathway, particularly cholesterol flux through liver to bile and feces. Attenuation of RCT and HDL efflux function, independent of HDL cholesterol levels, may contribute to atherosclerosis in chronic inflammatory states including obesity, metabolic syndrome, and type 2 diabetes. (Circulation. 2009;119:1135-1145.)

Key Words: atherosclerosis ■ cholesterol ■ inflammation ■ lipoproteins ■ macrophages

Reverse cholesterol transport (RCT) is the process by which cholesterol in peripheral cells (eg, lipid-laden foam cells) is effluxed onto circulating high-density lipoprotein (HDL) and transported back to the liver for secretion into bile and feces.1-2 Promotion of RCT is considered a major antiatherogenic function of HDL.3 Inflammation is proposed to impair HDL function and RCT. This may be of pathophysiologic significance because attenuation of RCT might contribute to atherosclerosis in chronic inflammatory states, including metabolic syndrome and type 2 diabetes. In the present study, we provide the first in vivo and ex vivo proof of concept that inflammation impairs RCT and does so at multiple steps in the RCT pathway.

Clinical Perspective p 1145

Previous studies in vitro and in vivo suggest that acute inflammation induces changes in HDL composition and metabolism that may impair RCT.4-6 These include induction of acute phase lipases that catabolize HDL phospholipids,5-8 increased HDL content of acute phase serum amyloid A (SAA) with displacement of apolipoprotein (apo) A-I,9 down-regulation of the hepatic HDL cholesterol receptor scavenger receptor class B type I (SR-BI),10 and reduced expression of hepatic transporters involved in excretion of cholesterol11 and bile acids.12 However, the integrated effect of in vivo inflammation on RCT and the relative impact on individual RCT steps have not been examined.

We employed subacute endotoxemia in the rodent macrophage-to-feces RCT model, previously described by our group,13-15 to assess the effects of inflammation on RCT in vivo. Briefly, this model tracks 3H-cholesterol from intraperitoneally injected J774 macrophages onto HDL in plasma and subsequent uptake by liver and clearance into bile and feces. Furthermore, ex vivo cholesterol efflux studies to HDL isolated from control and inflamed mice were performed to examine the capacity of acute phase HDL to accept cholesterol from macrophages. Finally, we performed translational studies in humans.
that extend the proof of concept by demonstrating that inflammation may also retard RCT in humans.

**Methods**

**Cell Culture**

**J774 Macrophage Preparation for RCT and Ex Vivo Cholesterol Efflux Studies**

RCT studies were performed as described previously.13–16 J774 macrophages were grown in suspension and incubated for 48 hours in labeling media containing acetylated low-density lipoprotein (LDL) (25 µg/mL) and 3H-cysteine (5 µCi/mL). Cells were washed, equilibrated, centrifuged, and resuspended in minimal essential media before intraperitoneal injection. For murine ex vivo efflux studies, J774 macrophages were labeled and loaded in a manner identical to that in in vivo studies (see Methods in the online-only Data Supplement). For human ex vivo efflux studies, we used a simple, established model of ABCA1 efflux17,18 unloaded J774 macrophages plus ACAT inhibitor (2 µg/mL) plus or minus cAMP (0.3 mmol/L).

In ex vivo cellular cholesterol efflux studies, plasma from mice was collected at 0, 6, 24, and 48 hours after lipopolysaccharide treatment (3 mg/kg SC). Serum from humans was collected at 4, 8, 12, and 24 hours after lipopolysaccharide treatment (3 ng/kg IV). ApoB-containing lipoproteins were removed from plasma/serum by polyethylene glycol precipitation.19 Ex vivo efflux from labeled macrophages to 2.8% HDL supernatant or minimal essential media control was measured over 4 hours. Efflux of 3H-cysteine was normalized to cholesterol content in supernatants.

**Human Macrophage Culture and In Vitro Cholesterol Efflux Studies**

Human monocytes, isolated from healthy volunteers, were purified by countercurrent elutriation and incubated in macrophage differentiation media for 7 days (RPMI 20% fetal bovine serum, 100 ng/mL macrophage colony-stimulating factor). Macrophages were loaded and labeled for 24 hours (RPMI, 10% fetal bovine serum, 50 µg/mL acetylated LDL, 6 µCi/mL 3H-cysteine), then washed, equilibrated, and treated with or without lipopolysaccharide (100 ng/kg IV). Cholesterol efflux to human apoA-I (20 µg/mL), HDL, (50 µg/mL), serum (2.5%), and minimal essential media control was assessed over a 4-hour period. Protein and mRNA expressions of cholesterol transporters were also assessed after 4-hour lipopolysaccharide treatment.

**Rodent In Vivo RCT Studies**

C57BL/6 mice, in metabolic cages (Tecniplast, USA, Inc) were fed Chow diet ad libitum. Our rationale for choice of route and dose of lipopolysaccharide and pilot study data are presented in the online-only Data Supplement. Anesthetized mice were injected subcutaneously with lipopolysaccharide (0.3 or 3 mg/kg) or PBS 4 hours before and 24 hours after intraperitoneal injection of labeled macrophages (~4.5 million cpm/2.3 million cells per mouse). Blood was collected, and liver and gall bladder were isolated and prepared for lipid, protein, and mRNA studies as described previously20,21 and in the online-only Data Supplement. 3H-label counts in plasma, liver, and feces are expressed as a percentage of total 3H-cysteine injected. The University of Pennsylvania Institutional Animal Care and Use Committee approved rodent studies, and mice were handled according to Institutional Animal Care and Use Committee guidelines.

**Human Endotoxemia Studies**

Healthy volunteers (n=20, 50% female, aged 18 to 40 years), without medical history, tobacco or prescription medication use, or laboratory abnormality were recruited from the Delaware Valley region.20,21 Whole blood samples were collected at indicated time points before and after intravenous infusion of 3 ng/kg US standard reference endotoxin (lot No. CC-RE-LOT-1+2, Clinical Center, National Institutes of Health), a dose that we have shown produces a robust inflammatory response.20 The University of Pennsylvania institutional review board approved the study, and written informed consent was provided.

**Laboratory Methods**

A description of general laboratory methods including lipoprotein analysis, quantitative real-time polymerase chain reaction, and immunoblot analysis are available in Methods in the online-only Data Supplement.

**Statistical Analysis**

Data are reported as mean±SEM. For mouse experiments with lipopolysaccharide and saline treatments over multiple time points, we performed 2-way repeated-measures ANOVA to test for differences in means. When the ANOVA test was significant, post hoc Bonferroni corrected t tests were applied. For comparison of data between lipopolysaccharide- and saline-treated groups at a single time point (liver, bile, feces, cellular efflux, and mRNA data), unpaired t tests were performed. Endotoxia effects over time in humans were tested by 1-way repeated-measures ANOVA, and, when significant, post hoc Bonferroni corrected t tests were performed comparing time points after lipopolysaccharide treatment to baseline. GraphPad Prism 5 (GraphPad Software Inc, San Diego, Calif) and Stata 9.0 software (Stata Corp, College Station, Tex) were used for statistical analyses. Statistical significance is presented as *P<0.05, **P<0.01, and ***P<0.001 in all figures.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

**Inflammatory and Lipoprotein Responses in Rodents After Endotoxin Challenge**

Subcutaneous administration of lipopolysaccharide (3 mg/kg) induced systemic and hepatic inflammation (Figure 1 in the online-only Data Supplement). Plasma levels of tumor necrosis factor-α increased transiently, whereas interleukin-6 had more sustained elevation (Figure IA and IB in the online-only Data Supplement). Hepatic mRNA levels of both cytokines were elevated at 6 hours (Figure IC in the online-only Data Supplement). No evidence of hepatotoxicity, as reflected in plasma alanine aminotransferase levels, was observed during endotoxia (Figure ID in the online-only Data Supplement).

As reported previously,4,22–24 lipopolysaccharide increased total plasma cholesterol levels (Figure IE in the online-only Data Supplement). Fast protein liquid chromatography (FPLC) profiles (48 hour) confirmed increased LDL cholesterol mass with lipopolysaccharide treatment (~2-fold) but minimal effect on HDL cholesterol mass (Figure IF in the online-only Data Supplement). Increased apoB protein in FPLC LDL fractions was observed (Figure IF and IG) and coincided with reduced hepatic expression of the LDL receptor (Table I in the online-only Data Supplement). An increase in cholesterol mass levels in the shoulder region between LDL and HDL peaks was also observed with lipopolysaccharide treatment (1.7-fold increase); these particles were apoA-I/apoB poor and apoE/SAA enriched (Figure IF and IG and Figure 2D). Increased HDL-associated apoE was observed despite reduced hepatic apoE mRNA levels during endotoxia (Table I in the online-only Data Supplement).

Initial RCT experiments were performed with 3 mg/kg lipopolysaccharide (Figures 1 to 3), a dose that induced significant lipoprotein changes as well as systemic and hepatic inflammation. Subsequently, we used a lower dose of
Figure 1. Lipopolysaccharide (LPS) (3 mg/kg SC) impairs macrophage 3H-cholesterol efflux into plasma in vivo. A, Plasma counts, measured over time across multiple studies, were normalized within each individual study, and percent reduction at indicated time points after lipopolysaccharide treatment is presented (n=54). A representative FPLC profile (1 of 3) is presented and revealed no change in cholesterol mass at 4 hours (B) and increased LDL and no change in HDL cholesterol mass at 48 hours after endotoxin (C). Lipopolysaccharide reduced 3H-cholesterol associated with HDL fractions at 4 hours (D) and 48 hours (E). Immunoblot analysis of FPLC fractions revealed a modest reduction in apoA-I and increase in apoB and apoE levels at 4 hours (F) and 48 hours (G) after lipopolysaccharide treatment (3 mg/kg) (*P<0.05, **P<0.01, ***P<0.001).
lipopolysaccharide (0.3 mg/kg; Figure 4) to determine the impact of low-grade inflammation on RCT.

Endotoxemia Reduced Macrophage $^3$H-Cholesterol Efflux to Plasma HDL In Vivo

The effect of lipopolysaccharide (3 mg/kg SC) on $^3$H-cholesterol movement from intraperitoneal macrophages into the plasma compartment was monitored over time. Analysis of pooled data across multiple studies (9 studies; n=6 mice per treatment group per study) showed a moderate but consistent reduction in plasma counts at 4 and 24 hours with no difference observed at 48 hours (Table, Figure 1A).

FPLC profiles of plasma from 3 separate experiments were analyzed to assess the distribution of cholesterol mass and label across lipoproteins during endotoxemia. In control mice, the majority of counts ($\approx 65\%$) were found in HDL fractions. At 4 hours, counts in HDL fractions were reduced by 61% with lipopolysaccharide and remained attenuated at 24 hours ($\approx 44\%$) and 48 hours ($\approx 16.5\%$) (Figure 1D and 1E). In contrast, little change in HDL cholesterol mass was observed with lipopolysaccharide at 4 hours (1.17-fold control), 24 hours (1.07-fold control), and 48 hours (1.04-fold control) (Figure 1B and 1C). There was a reduction in the ratio of cholesterol counts/mass, ie, the rate of enrichment of

---

**Figure 2.** HDL remodeling during endotoxemia impairs HDL acceptor function. A, Endotoxemia (3 mg/kg) induced hepatic SAA mRNA. B, Plasma SAA protein levels markedly increased after lipopolysaccharide (LPS) treatment (n=6). FPLC profiling demonstrated that the majority of SAA was associated with HDL fractions at 4 hours (C) and 48 hours (D); further SAA was detectable in the apoE-rich shoulder region at 48 hours (fractions 22 to 28). E, Ex vivo cholesterol efflux from $^3$H-cholesterol-labeled, acetylated LDL–loaded J774 macrophages to HDL isolated from lipopolysaccharide-treated mice (n=5) was reduced at all time points after lipopolysaccharide treatment ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).
Figure 3. Endotoxin impairs \(^3\text{H}\)-cholesterol movement through liver to bile and feces. A, No significant effect on liver counts was observed at 48 hours after lipopolysaccharide (LPS) treatment. B, Reduced mRNA expression of ABCA1 and SR-BI was observed across multiple studies with no effect on ABCG1 expression. C, Lipopolysaccharide markedly reduced bile counts at 48 hours. This coincided with reduced mRNA expression of hepatic transporters ABCG5, ABCG6, and ABCB11 and the bile-acid synthesis enzyme CYP7A1 (D), as well as reduced \(^3\text{H}\)-cholesterol levels in feces (n=54) (E). F, Immunoblot analysis of liver membrane lysates revealed no change in SR-BI, ABCA1, and ABCG1 protein expression but a marked reduction in ABCG5/8 heterodimer expression at 24 hours. Densitometry data, normalized to \(\beta\)-actin, are shown for SR-BI and ABCG5/8 heterodimer (*\(P\leq 0.05\), **\(P\leq 0.01\), ***\(P\leq 0.001\)).
HDL with new ¹³C-cholesterol from macrophages after lipopolysaccharide treatment (Table II in the online-only Data Supplement). Analysis of FPLC fractions revealed decreased apoA-I and increased apoE and SAA content in HDL fractions after lipopolysaccharide treatment (Figure 1F and 1G and Figure 2C and 2D). A marked induction in hepatic SAA mRNA (Figure 2A) coincided with increased plasma protein levels (Figure 2B). In summary, during endotoxemia we
found that (1) HDL cholesterol mass was unchanged but HDL apoA-I content was reduced; (2) HDL particles were enriched with SAA and apoE; and (3) there was reduced 3H-cholesterol movement from macrophages to HDL.

Endotoxemia Impairs Murine HDL Efflux Capacity Ex Vivo

These findings suggest that lipopolysaccharide reduced 3H-cholesterol transfer from macrophages to remodeled HDL particles in vivo. Therefore, we determined, ex vivo, the capacity of the inflammatory HDL fraction (2.8% supernatant after polyethylene glycol precipitation of apoB) to promote 3H-cholesterol efflux from loaded J774 macrophages. Efflux from macrophages to HDL supernatant collected at 6, 24, and 48 hours after lipopolysaccharide treatment (from a 3-mg/kg SC non-RCT study) was significantly reduced compared with saline-treated control (Figure 2E). These studies support the concept that during inflammation, alteration in HDL structure/composition impairs HDL acceptor function in vivo.

Endotoxemia Markedly Impairs Hepatic to Bile and Fecal 3H-Cholesterol Secretion

We assessed the effects of inflammation on later steps in the RCT pathway involving hepatic 3H-cholesterol uptake and flux through liver to bile and feces. Analysis of hepatic counts across multiple studies revealed a nonsignificant reduction (−13.5±6.2%) in counts at 48 hours with lipopolysaccharide (Figure 3A and Table). Despite a significant reduction in hepatic SR-BI mRNA during endotoxemia (Figure 3B), little change in SR-BI protein was observed (Figure 3F). Little difference in hepatic ABCG1 and ABCA1 protein levels was observed at any time point, consistent with minimal effects on hepatic mRNA of these transporters (Figure 3B and 3F).

The most pronounced effect of endotoxemia on the RCT pathway was on 3H-cholesterol secretion from liver to bile and feces (Table, Figure 3). At 48 hours, lipopolysaccharide markedly reduced bile counts (−56.3±4.5%) coincident with reduced mRNA levels for ABCG5, ABCG8, and ABCB11 transporters and the bile-acid synthesis enzyme CYP7A1 (Figure 3C and 3D). Effects on transporter mRNAs were observed as early as 6 hours after lipopolysaccharide treatment: ABCG5 (−84.1±1.5%), ABCG8 (−91.1±1.7%), and ABCB11 (−88.9±2.3%) (n=5; P<0.001 for all). A reduction in ABCG5/8 heterodimer (150 kDa) protein levels was observed at all time points, consistent with reduced mRNA levels (Figure 3F, 24-hour blots presented).

A consistent reduction in fecal counts was also observed after lipopolysaccharide treatment (−62.1±5.7%) (Table, Figure 3E). Lipopolysaccharide had similar effects on fecal free cholesterol (−71.9±4.8%) and bile-acid (−65.2±5.9%) levels (pooled data representative of 3 studies; n=6 per treatment group per study; P<0.001).

Low-Dose Endotoxin Selectively Blocks Hepatic Cholesterol and Bile-Acid Secretion

Because the effects of a moderate subcutaneous lipopolysaccharide dose on bile counts were dramatic, we hypothesized that mild “subclinical” inflammation with low-dose lipopolysaccharide might attenuate liver to bile 3H-cholesterol even in the absence of significant effects on lipoprotein levels or HDL functionality. Low-dose lipopolysaccharide (0.3 mg/kg SC) had minimal effects on plasma tumor necrosis factor-α levels (P=0.56) (Figure 4A), induced modest increase in plasma SAA levels (76.3±21.1 to 167.4±11.0 mg/dL at 48 hours; n=6; P<0.01), but had no effect on serum lipoprotein FPLC profiles (Figure 4C). Low-dose lipopolysaccharide did not reduce 3H-cholesterol plasma counts (Figure 4B), had no impact on HDL cholesterol counts/mass ratio (1.1) (Figure 4C and 4D), and did not reduce liver counts (Figure 4E). Nonetheless, low-dose lipopolysaccharide significantly reduced bile (−36%) and fecal counts (−69%) (Figure 4F and 4G), which correlated with reduced ABCG5 (−23%), ABCG8 (−37%), and ABCB11 (−44%) mRNAs (Figure 4H).

Endotoxemia Modulates the RCT Pathway in Humans

We performed proof of concept studies in humans to establish whether experimental endotoxemia modulates components of human RCT in vivo. As reported previously, endotoxin (3 ng/kg IV) induced a short flu-like illness in humans with acute and transient increases in inflammatory cytokines that resolved within 6 to 8 hours. 20 A significant and sustained elevation of C-reactive protein was evident after lipopolysaccharide treatment (Figure 5D). Minimal reductions in plasma HDL cholesterol and apoA-I were observed (Figure 5A and 5B). There were, however, substantial decreases in plasma (Figure 5C) and HDL (−27.8±8.1% at 24 hours; n=20; P<0.005) phospholipids, whereas plasma (Figure 5D) and HDL-associated (38.3±3.7-fold increase at 24 hours; n=20; P<0.001) SAA increased dramatically. Furthermore, compared with baseline there was a marked and progressive reduction in the ex vivo capacity of human HDL (baseline, 6, 24, and 48 hours) to promote 3H-cholesterol efflux from J774 macrophages after lipopolysaccharide treatment (Figure 5E). These findings are consistent with inflammatory-mediated remodeling of HDL and impaired RCT in humans during inflammation.

Table. Effect of Lipopolysaccharide (3 mg/kg) on RCT

<table>
<thead>
<tr>
<th>Counts</th>
<th>Percent Change</th>
<th>ANOVA F Statistic</th>
<th>P (Compared With Saline Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma 4 h</td>
<td>↓31.6±3.5%</td>
<td>P&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Plasma 24 h</td>
<td>↓19.5±4%</td>
<td>F=24.33</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Plasma 48 h</td>
<td>↓4.4±5.3%</td>
<td>P=0.96</td>
<td></td>
</tr>
<tr>
<td>Liver 48 h</td>
<td>↓13.5±6.2%</td>
<td>N/A</td>
<td>P=0.47</td>
</tr>
<tr>
<td>Bile 48 h</td>
<td>↓56.3±4.5%</td>
<td>N/A</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Feces 0–48 h</td>
<td>↓62.1±5.7%</td>
<td>N/A</td>
<td>P&lt;0.005</td>
</tr>
</tbody>
</table>

Percent change in 3H-cholesterol counts during endotoxemia compared with saline control (pooled data from 9 experiments with 6 mice per group; n=54 mice per group). Data are reported as mean±SEM. For mouse experiments with lipopolysaccharide and saline treatments over multiple time points, we performed 2-way repeated-measures ANOVA to test for differences in means; when significant, post hoc Bonferroni corrected t tests were applied. For comparison of data between lipopolysaccharide and saline groups at a single time point (liver, bile, and feces data), unpaired t tests were performed.
Figure 5. Human acute phase HDL has reduced acceptor capacity for macrophage \(^3\)H-cholesterol ex vivo. Human experimental endotoxemia (3 ng/kg IV bolus) had nonsignificant effects on plasma levels of HDL cholesterol (HDL-C) (A) \((F=0.39, P=0.91)\) and moderately decreased apoA-I (B) at later time points. A reduction in plasma phospholipid (C) and a marked induction of plasma SAA levels and high-sensitivity C-reactive protein (CRP) (D) was observed as early as 6 hours after lipopolysaccharide (LPS) \((n=20)\). E, Ex vivo \(^3\)H-cholesterol efflux from J774 macrophages to acute phase HDL \((2.8\% \text{ polyethylene glycol supernatant})\) was reduced at 24 hours after lipopolysaccharide treatment \((n=20)\). F, \(^3\)H-cholesterol efflux from lipopolysaccharide-treated \((100 \text{ ng/mL for 4 hours})\) human macrophages in vitro was reduced to apoA-I \((20 \mu\text{g/mL})\) and serum \((2.5\%)\) but not to HDL\(_3\) \((50 \mu\text{g/mL})\). G, Lipopolysaccharide reduced mRNA expression of ABCA1, ABCG1, and SR-BI in human macrophages \((\text{cells derived from 3 people})\). H, Immunoblot analysis revealed a marked reduction in ABCA1, a moderate reduction in SR-BI, and little change in ABCG1 protein levels \((^*P<0.05, ^{**}P<0.01, ^{***}P<0.001)\).
Endotoxin Impairs Human Macrophage Efflux Function In Vitro

Inflammation may also directly impair human macrophage cholesterol efflux, the first step of the RCT pathway. Lipopolysaccharide (100 ng/mL for 4 hours) reduced in vitro 3H-cholesterol efflux from human monocyte-derived macrophages to apoA-I (−19.6%) and serum (−18.3%) with no effect on efflux to HDL₃ (−3.1%) (Figure 5F). Significant reductions in cholesterol transporters ABCA1 and ABCG1 and SR-BI mRNA levels were apparent (Figure 5G). Immunoblot analysis revealed a marked reduction in ABCA1, a moderate reduction in SR-BI, and little change in ABCG1 protein levels (Figure 5H).

Discussion

We demonstrate that acute inflammation retards RCT in vivo and provide evidence that this atherogenic response may occur in humans during inflammatory syndromes. Endotoxemia has a broad and integrated impact on RCT, attenuating several steps including macrophage cholesterol efflux, HDL acceptor function, and hepatic to bile/fecal cholesterol elimination. The profound impact on cholesterol secretion into bile, even with low doses of lipopolysaccharide, provides a novel insight into a major inflammatory regulation of the final steps of RCT. Overall, our studies suggest a substantial attenuation of atheroprotective RCT, with apparent conservation of body cholesterol stores, during inflammation.

Our findings showed reduced plasma 3H-cholesterol counts at early time points after lipopolysaccharide treatment with less pronounced effects at later time points. Because plasma counts are a single integrative measure of RCT, it is difficult to draw conclusions about effects on individual RCT processes solely from changes in plasma counts. Different components of RCT can influence plasma counts in opposite directions; impaired efflux from macrophages and reduced acceptor capacity of HDL reduce plasma counts, whereas reduced flux through liver to bile and feces may increase counts. Notably, in the setting of reduced RCT, plasma counts of SR-BI-deficient mice are increased because of reduced hepatic clearance of HDL cholesterol. Indeed, our data suggest that inflammation may reduce the flux of 3H-cholesterol from plasma through liver because of a marked decrease in clearance from liver to bile while also impairing macrophage efflux and HDL acceptor function. These opposing influences may account for the modest effect on 48-hour plasma counts.

We performed in vitro studies examining the direct impact of lipopolysaccharide on macrophage cholesterol efflux using a foam cell model of cholesterol-loaded, primary human monocyte-derived macrophages. Lipopolysaccharide impaired cholesterol efflux to human apoA-I and serum coincident with reduced expression of ABCA1. There was little change in ABCG1 protein, perhaps accounting for the lack of effect on macrophage efflux to HDL₃, an acceptor for ABCG1-mediated efflux. Despite reductions in SR-BI mRNA and protein, the lack of effect of lipopolysaccharide on HDL₃ efflux also argues against a significant role for SR-BI in human macrophage–foam cell efflux, as has been suggested by recent studies in rodents. These findings suggest that inflammation may chronically impair ABCA1-mediated cholesterol efflux from arterial foam cells, thus accelerating atherosclerosis.

Reduced 3H-cholesterol in plasma may also reflect reduced capacity of inflammatory HDL particles to efficiently accept cholesterol from macrophages. HDL particles undergo remodeling during acute inflammation through activation of lipases such as secretory phospholipase A₂, and endothelial lipase, induction of and HDL association with SAA, attenuation of lecithin cholesterol acyltransferase (and cholesterol ester transfer protein activity in humans), as well as oxidant modification of apoA-I. In fact, such changes have been shown to modify HDL efflux and anti-inflammatory functions. In our in vivo rodent studies, the marked reduction of HDL 3H-cholesterol/cholesterol mass suggests an inflammatory-mediated loss of HDL acceptor function in vivo coincident with impaired RCT. In parallel, our ex vivo studies of HDL function demonstrated that inflammatory remodeling of HDL impairs its capacity to serve as an acceptor for macrophage ABCA1 cholesterol efflux.

Van der Westhuyzen and colleagues have shown that SAA can accept cholesterol from ABCA1 and SR-B1 efflux pathways and, contrary to our findings, demonstrated increased efflux to HDL from mice overexpressing SAA and acute phase HDL. Differences in cell models (J774 macrophages versus hepatocytes and CHO cells overexpressing SR-B1) and differences in HDL preparation may account for discrepant findings. Our studies do not directly address SAA efflux function, whereas Van der Westhuyzen et al did not assess the impact of inflammation on other aspects of HDL function or on RCT in vivo. It is not clear that increased HDL-SAA in vivo improves HDL acceptor function. For example, SAA may displace apoA-I from HDL and, in conjunction with other documented inflammatory HDL changes, may not improve HDL efflux functions. In fact, our mouse in vivo RCT and ex vivo HDL efflux data as well as our human HDL ex vivo efflux studies strongly suggest that the integrated inflammatory effect is to retard multiple steps of RCT in vivo including HDL cholesterol acceptor function.

During rodent endotoxemia, we observed increased numbers of larger HDL particles that were apoA-I poor and apoE/SAA enriched. Increased HDL-associated apoE has been reported previously and is likely attributable to reduced clearance via the LDL receptor rather than increased production as we and others found reduced hepatic apoE mRNA levels during endotoxemia. We also found that lipopolysaccharide increased total cholesterol and apoB levels, as has been reported in rodents. Mechanisms of apoB lipoprotein changes include reduced LDL clearance due to downregulation of LDL receptor and upregulation of PCSK9 and increased 3-hydroxy-3-methylglutaryl coenzyme A reductase activation. Our findings, however, suggest that increased apoB plays a trivial role, if any, in modulating RCT changes during inflammation.

Despite species differences in lipoprotein metabolism, several inflammatory-mediated changes in HDL structure, composition, and efflux function appear consistent across human and rodent species. In humans, endotoxin reduced plasma and HDL...
phospholipids, possibly as a result of activation of inflammatory HDL lipases, endothelial lipase, and secretory phospholipase A₂.\textsuperscript{31,45} We recently demonstrated that reduced HDL phospholipids coincided with induction of endothelial lipase during endotoxemia.\textsuperscript{32} Despite only modest changes in human HDL cholesterol and apoA-I levels, endotoxin markedly increased plasma and HDL-associated SAA. Furthermore, the functional capacity of human acute phase HDL to efficiently efflux cholesterol from macrophages ex vivo was impaired, in concordance with our rodent studies. Our human studies provide evidence for consistent effects, across species, of endotoxin on HDL function.

In rodent studies, the greatest impact of lipopolysaccharide was on movement of \(^3\)H-cholesterol through liver to bile and feces, indicating that inflammation targets hepatic RCT-related lipoprotein metabolism. Despite the consistent reduction in hepatic SR-BI mRNA expression, little difference in SR-BI protein levels and hepatic \(^3\)H-cholesterol counts was observed, suggesting that hepatic cholesterol uptake is not affected during inflammation. In contrast, the striking reduction in bile and fecal counts suggests attenuation of cholesterol elimination from liver to bile. A remarkable and consistent finding was early and sustained suppression of the biliary cholesterol transporters to bile. A remarkable and consistent finding was early and sustained suppression of the biliary cholesterol transporters to bile. A remarkable and consistent finding was early and sustained suppression of the biliary cholesterol transporters to bile.

The endotoxia model used in our rodent and human studies provides proof of principle that inflammation attenuates RCT while being broadly relevant to human inflammatory pathophysiology, especially infections, sepsis, and acute rheumatologic disorders. Although subacute in nature, the low-dose rodent studies may be of relevance to chronic conditions associated with hepatic inflammation including nonalcoholic steatohepatitis,\textsuperscript{46} hepatic insulin resistance, and metabolic syndrome.\textsuperscript{47,48} We emphasize, however, the need for additional work to assess the specific impact on RCT of chronic low-grade human inflammation in obesity, metabolic syndrome, and atherosclerosis.

**Conclusions**

We present the first in vivo functional evidence to support the hypothesis that inflammation impairs RCT and does so at numerous steps in the pathway from initial macrophage efflux to HDL acceptor function and the final step of cholesterol flux through liver to bile and feces. This study strengthens the hypothesis that impaired RCT may be an important link between the low-grade inflammation of insulin-resistant conditions and the development and acceleration of atherosclerosis.

**Acknowledgments**

We would like to gratefully acknowledge Aisha Wilson, Maosen Sun, Edwige Edouard, Anna DiFiorio, Linda Morrell, and Leticia Prusciono for their technical expertise.

**Sources of Funding**

This work was supported by a Clinical and Translational Science Award (RFA-RM-06-002) from the National Center for Research Resources, RO1 HL-073278 (Dr Reilly), P50 HL-083799-SCCOR (Dr Reilly), and the Alternative Drug Discovery Initiative award to the University of Pennsylvania from GlaxoSmithKline.

**Disclosures**

Dr Reilly is the recipient of research grants from GlaxoSmithKline and Merck Research Laboratories. The other authors report no potential conflicts of interest.

**References**

 Reverse cholesterol transport (RCT) is the process by which cholesterol can be transported from peripheral cells, such as lipid-laden macrophages in atherosclerotic lesions, to the liver for secretion into bile and feces. Promotion of RCT is considered the key atheroprotective function of HDL. RCT is a complex pathway and involves several regulated steps including macrophage cholesterol efflux, HDL cholesterol acceptor function, cholesterol uptake into the liver, and ultimately cholesterol elimination into bile and feces. Systemic inflammation, a hallmark of chronic atherosclerosis, is thought to retard RCT, thus promoting atherosclerosis. However, in vivo evidence is lacking to support this concept. In the present article, we show for the first time that acute inflammation can impair multiple steps of RCT in vivo. These translational proof-of-concept studies demonstrate that inflammation retards cholesterol efflux from human macrophages, reduces HDL cholesterol acceptor function ex vivo and in vivo in rodents, and blocks flux of cholesterol from liver to bile by downregulating specific biliary transporters, eg, ABCG5 and ABCG8. This work supports the concept that chronic inflammatory states, including obesity, metabolic syndrome, and type 2 diabetes, impaired RCT may contribute to atherosclerosis and its clinical consequences.
Inflammation Impairs Reverse Cholesterol Transport In Vivo
Fiona C. McGillicuddy, Margarita de la Llera Moya, Christine C. Hinkle, Michelle R. Joshi, Elise H. Chiquoine, Jeffrey T. Billheimer, George H. Rothblat and Muredach P. Reilly

Circulation. 2009;119:1135-1145; originally published online February 16, 2009;
doi: 10.1161/CIRCULATIONAHA.108.810721
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/119/8/1135

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2009/02/13/CIRCULATIONAHA.108.810721.DC1
http://circ.ahajournals.org/content/suppl/2009/02/27/CIRCULATIONAHA.108.810721.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/
SUPPLEMENT MATERIAL

Supplement Methods

Materials

[1,2 \(^{3}\)H]-cholesterol (Perkin-Elmer Analytical Sciences, Boston, MA), LPS (Sigma Aldrich, St. Louis, MO), Macrophage Colony Stimulating Factor (M-CSF) (Peprotech, Rocky Hill, NJ), RPMI 1640 (GIBCO, Invitrogen, Eugene, OR), RNAlater (Ambion, Austin, TX) and fetal bovine serum (FBS) (HyClone, Logan, UT) were obtained commercially. J774 macrophages were obtained from American Type Culture Collection (ATCC, Manassas, Ca). ACAT inhibitor was donated from Pfizer Pharmaceuticals, Groton, CT. Wild-type female C57BL/6 mice (8-12 weeks old) were purchased from Jackson Laboratories (Bar Harbour, Maine). Human monocytes were obtained from the Human Immunology Core (HIC) of the Penn Center for AIDS Research (CFAR). All other reagents purchased from Sigma Aldrich (St Louis, MO). Human endotoxin studies were performed at Clinical and Translational Research Center (CTRC) at the University of Pennsylvania.

Methods

J774 macrophage preparation for RCT studies

For murine RCT studies and ex vivo efflux studies, J774 macrophages were labeled and loaded identically - 48h in labeling media containing acetylated LDL (25µg/mL) and \(^{3}\)H-cholesterol (5µCi/mL). Under these experimental conditions (25µg/ml acLDL loaded for 48h time), we have found that loaded J774 macrophages have foam-cell like
characteristics (total cholesterol of 87.1 ± 4.5 µg/mg protein; 47.9 ± 5.1 % cholesterol ester, analysis of cells from n=4 studies) and that J774 macrophages express abundant ABCA1 protein (Supplement figure 2a) and support robust efflux to the major ABCA1 acceptor apoA-I (Supplement figure 2b), consistent with work by Favari et al\textsuperscript{1}.

**J774 macrophage preparation for human HDL\textit{ ex vivo} efflux studies**

For human \textit{ex vivo} efflux studies, the established J774 macrophage model (non acLDL loaded, ± cAMP (0.3mM), + ACAT inhibitor (2µg/ml)\textsuperscript{2,3} ) maximizes ABCA1 expression and provides a ready pool of free cholesterol (ACAT inhibition) for short term efflux studies which are not confounded by variation in macrophage cholesterol esterification and hydrolysis. In this model, efflux values from non-cAMP treated cells are subtracted from efflux to cAMP treated cells to measure ABCA1-dependent efflux.

**In vivo RCT Studies**

*Rationale for choice of LPS dose and route of administration*

Overall, we chose doses and routes of LPS administration based on published literature (mouse and human)\textsuperscript{4-10}, pilot studies (mouse) and experimental needs (mouse) in order to elicit a reproducible, non-lethal, non-toxic acute inflammatory response. In rodents, we wished to employ a sub-acute model that induced a slower rate of endotoxin release into the blood-stream and therefore eliminated the intravenous (IV) route of administration (ROA). Because we reserve the intraperitoneal (IP) cavity for labeled macrophage administration, we chose the subcutaneous (SQ) route. Given the paucity of published data\textsuperscript{11 4-7}, we performed pilot studies comparing inflammatory and lipid responses across
different LPS administrations, IV LPS (0.1 and 0.3 mg/kg) vs. SQ LPS (3 and 10 mg/kg). All doses produced robust increases in plasma TNFα levels (Supplement figure 3A). Notably, peak inflammatory responses with 3mg/kg SQ were comparable to a much lower IV dose (0.1 mg/kg). FPLC profiles of plasma at 48h revealed a marked induction in LDL-C peak with all LPS doses and ROA with little change in HDL-C mass peak (Supplement figure 3B). We chose 3 mg/kg SQ LPS for our initial studies because this produced a robust, non-toxic (no increase in liver enzymes), acute systemic inflammatory response accompanied by changes in plasma lipoproteins (Supplement figure 1A-F). We chose a lower dose LPS (0.3 mg/kg SQ) in rodents in order to assess the effect of modest hepatic inflammation (~2 fold elevation in plasma levels of hepatic derived SAA) on RCT (especially on the final liver to bile steps) in the setting of low grade systemic inflammation (<3 fold increase in plasma cytokines).

Parenthetically, LPS at 3mg/kg SQ induced ~20 fold peak-increase in plasma cytokines and ~112 fold increase in plasma SAA (Figure 2B) levels in mice, which is of the same order of magnitude as the increases in plasma cytokines (~50 fold) and SAA (~39 fold) (Figure 5) observed with LPS 3ng/kg IV in humans. In contrast, low dose LPS (0.3 mg/kg SQ) in rodents induced ~2-fold increase in plasma cytokines (Figure 4) and ~2 fold increase in plasma SAA.

**Sample Processing for rodent in vivo RCT studies:** Blood, collected via the retro-orbital plexus from anesthetized mice, was centrifuged for plasma supernatant. At 48h mice were sacrificed by cervical dislocation, liver and gall bladder isolated; liver portions (50mg) snap-frozen for lipid and RNA extraction. Tissue lipids were extracted according
to Bligh and Dyer procedure and $^3$H-cholesterol levels in plasma, bile, and extracted lipids measured by liquid scintillation counting (LSC). Feces (0-48h) were collected and soaked overnight in ddH$_2$O (100mg feces/1mL), ethanol added, samples homogenized and free cholesterol isolated by hexane extraction. The aqueous phase was adjusted to pH ≤ 1 for bile acid extraction with ethyl acetate. $^3$H-label counts in plasma, liver and feces are expressed as a percentage of total $^3$H-cholesterol injected.

General Laboratory Methods

**Lipoprotein Analysis and Enzyme Linked Immunosorbent Assays (ELISA):** Pooled plasma from mice (150μL, N=6) was separated by Fast Protein Liquid Chromatography (FPLC) (Amersham Pharmacia Biotech). Plasma and FPLC fraction total cholesterol levels were measured enzymatically (Wako Pure Chemical Industries). Counts in FPLC fractions (cpm/100μl) were measured by LSC. In humans, following ultracentrifugation, plasma lipoproteins, apolipoproteins, phospholipids and hsCRP were measured enzymatically (Wako Diagnostics, Richmond, VA) on a Hitachi 912 analyzer (Roche Diagnostics, Basel, Switzerland). Levels of murine TNF$\alpha$ and IL6 (R&D systems, Minneapolis, MN) and SAA (Life Diagnostics, West Chester, PA) in plasma and FPLC fractions and human plasma SAA (BioSource, Invitrogen, Eugene, OR) were measured by ELISA.

**RNA isolation and quantitative Real-time PCR analysis:** Liver tissue was homogenized in Trizol using a tissue lyser (Qiagen), RNA (500ng) isolated, and reverse transcribed using an Applied Biosystems High Capacity cDNA archive kit. Levels of mRNAs, using
Applied Biosystems primers, probes and 7300 sequence detector, were assessed by quantitative Real-Time PCR and normalized to β-actin levels.

**Immunoblot analysis:** FPLC fractions were reduced, separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes and probed with apoA-I, apoE and apoB primary antibodies (BIODESIGN International, Meridian Life Science, Maine). Blots were visualized by chemiluminescence (ECL plus kit, GE Healthcare). Liver tissue (50mg) was homogenized in PBS (+protease inhibitors) and centrifuged at low speed (2000xg for 10min). Liver membrane proteins were isolated from supernatant by high-spin ultracentrifugation (56,806xg for 1h at 4°C). The remaining pellet was resuspended in lysis buffer, quantitated by bicinchoninic acid (BCA) assay and equal quantities (10µg/lane) separated by SDS-PAGE electrophoresis prior to transferring to nitrocellulose membranes. Membranes were blocked and probed with ABCA1, ABCG1, SR-BI, ABCG8 and β-actin antibodies (Novus Biologicals, Littleton, CO).
**Supplement Tables**

**Supplement Table 1.** Fold change in hepatic mRNA expression of genes at 48h post LPS (3mg/kg, SQ) as assessed by Real-Time PCR (n=6 mice, data presented as mean ± SEM).

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Saline (1.06 ± 0.17)</th>
<th>LPS (3mg/kg) (0.41 ± 0.06)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I</td>
<td>1.06 ± 0.17</td>
<td>0.41 ± 0.06</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>ApoB</td>
<td>1.04 ± 0.15</td>
<td>0.65 ± 0.04</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>ApoE</td>
<td>1.08 ± 0.22</td>
<td>0.41 ± 0.06</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>LDLR</td>
<td>1.01 ± 0.07</td>
<td>0.47 ± 0.04</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>LXR</td>
<td>1.01 ± 0.07</td>
<td>0.78 ± 0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>LCAT</td>
<td>1.01 ± 0.09</td>
<td>0.69 ± 0.02</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>HL</td>
<td>1.05 ± 0.17</td>
<td>0.45 ± 0.08</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>PLTP</td>
<td>1.07 ± 0.18</td>
<td>0.38 ± 0.03</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>PON1</td>
<td>1.06 ± 0.15</td>
<td>0.62 ± 0.04</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

* For comparison of Real-Time data between LPS and saline groups unpaired t-tests were performed.
Supplement Table 2. Endotoxin Reduces HDL Cholesterol Acceptor Function in vivo

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>4 Hours</th>
<th>24 Hours</th>
<th>48 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n=3)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>LPS (3mg/kg) (n=3)</td>
<td>0.61 ± 0.14</td>
<td>0.78 ± 0.13</td>
<td>0.85 ± 0.16</td>
</tr>
</tbody>
</table>

*Data representative of 3 independent studies (n=6 mice per treatment group per study).

Plasma from control or LPS treated mice was pooled in each individual study and separated by FPLC. Data presented as mean ± SEM.
Supplement Figures

Supplement Figure 1: Subcutaneous endotoxin (3mg/kg SQ) induces a robust systemic inflammatory response in vivo

Increased plasma protein levels of (A) TNFα and (B) IL6 during endotoxemia (3mg/kg SQ). (C) Induction of hepatic IL6 and TNFα mRNA following LPS. (D) Plasma levels of the liver enzyme, alanine aminotransferase (ALT) were measured enzymatically (Wako Diagnostics, Richmond, VA) on a Hitachi 912 analyzer (Roche Diagnostics, Basel, Switzerland). There was no evidence for increase in ALT at any time point post-LPS (n=3 mice per group). (E) Total cholesterol levels in mice following endotoxin (n=6). (F) FPLC cholesterol profiles at 48h. A marked increase in the LDL-cholesterol peak was observed with no change in HDL-cholesterol mass peak. An increase in cholesterol in the “shoulder” region between LDL and HDL peaks (Fractions 22-28) was also observed. Statistical significance is presented as *p<0.05, **p<0.01 and ***p<0.001 in all figures.

Supplement Figure 2: AcLDL-loaded J774 macrophages express abundant ABCA1 and support robust efflux to ApoA-I

(A) J774 macrophages were treated ± acLDL (20µg/ml) ± cAMP (0.3mM) for 24h prior to harvesting in RIPA buffer. Immunoblot analysis revealed an increase in ABCA1 protein levels with both acLDL (20µg/ml) and cAMP (0.3mM) treatment. (B) J774 macrophages were loaded and labeled for 24h (RPMI, 50µg/ml gentamicin, 1µCi/ml 3H-cholesterol and 50µg/ml acLDL) prior to equilibration for a further 24h (RPMI, 50µg/ml gentamicin and 0.2 % BSA). Cells were washed and efflux to apoA-I (20µg/ml), HDL3
(25\(\mu\)g/ml) or media alone (MEM) was monitored over a 4h efflux period. Loaded J774 macrophages support robust efflux to ApoA-I and HDL3 compared with efflux to MEM (n=4, *p<0.05, **p<0.01 and ***p<0.001).

**Supplement Figure 3: Pilot studies of route and dose of LPS administration**

(A) The effect of different doses of LPS administered SQ and IV on inflammatory markers and lipoprotein changes were compared. All doses increased plasma TNF\(\alpha\) levels; however peak effects of 3mg/kg LPS SQ were similar to a much lower IV dose (0.1 mg/kg) (n=4, *p<0.05, **p<0.01 and ***p<0.001). (B) FPLC profiles of plasma at 48h revealed a marked induction in LDL-C peak with all LPS doses and little change in HDL-C mass peak.
References


Supplement Figure 1

A) TNFα (pg/ml)

B) IL6 (pg/ml)

C) Hepatic cytokine (Fold change)

D) ALT (U/L)

E) Total cholesterol (mg/dL)

F) Cholesterol (mg/dL)
Supplement Figure 2

A

acLDL (20µg/ml):
- - + +
cAMP (0.3mM):                      - + - +

B

% Efflux/4h

MEM ApoAI HDL3
Acceptor

0.0 0.5 1.0 1.5 2.0 2.5

*** **
Supplement Figure 3

A

![Bar graph showing TNFα levels](image)

- Saline
- 3mg/kg SQ
- 10mg/kg SQ
- 0.1mg/kg IV
- 0.3mg/kg IV

B

![Line graph showing Cholesterol levels](image)

- Control
- IV (0.1mg/kg)
- IV (0.3mg/kg)
- SQ (3mg/kg)
- SQ (10mg/kg)
Supplement Methods

Materials

\[1,2^{3}H\]-cholesterol (Perkin-Elmer Analytical Sciences, Boston, MA), LPS (Sigma Aldrich, St. Louis, MO), Macrophage Colony Stimulating Factor (M-CSF) (Peprotech, Rocky Hill, NJ), RPMI 1640 (GIBCO, Invitrogen, Eugene, OR), RNAlater (Ambion, Austin, TX) and fetal bovine serum (FBS) (HyClone, Logan, UT) were obtained commercially. J774 macrophages were obtained from American Type Culture Collection (ATCC, Manassas, Ca). ACAT inhibitor was donated from Pfizer Pharmaceuticals, Groton, CT. Wild-type female C57BL/6 mice (8-12 weeks old) were purchased from Jackson Laboratories (Bar Harbour, Maine). Human monocytes were obtained from the Human Immunology Core (HIC) of the Penn Center for AIDS Research (CFAR). All other reagents purchased from Sigma Aldrich (St Louis, MO). Human endotoxin studies were performed at Clinical and Translational Research Center (CTRC) at the University of Pennsylvania.

Methods

J774 macrophage preparation for RCT studies

For murine RCT studies and \textit{ex vivo} efflux studies, J774 macrophages were labeled and loaded identically - 48h in labeling media containing acetylated LDL (25\(\mu\)g/mL) and \(^3\)H-cholesterol (5\(\mu\)Ci/mL). Under these experimental conditions (25\(\mu\)g/ml acLDL loaded for 48h time), we have found that loaded J774 macrophages have foam-cell like
characteristics (total cholesterol of 87.1 ± 4.5 µg/mg protein; 47.9 ± 5.1 % cholesterol ester, analysis of cells from n=4 studies) and that J774 macrophages express abundant ABCA1 protein (Supplement figure 2a) and support robust efflux to the major ABCA1 acceptor apoA-I (Supplement figure 2b), consistent with work by Favari et al.1.

**J774 macrophage preparation for human HDL ex vivo efflux studies**

For human ex vivo efflux studies, the established J774 macrophage model (non acLDL loaded, ± cAMP (0.3mM), + ACAT inhibitor (2µg/ml)2-3 ) maximizes ABCA1 expression and provides a ready pool of free cholesterol (ACAT inhibition) for short term efflux studies which are not confounded by variation in macrophage cholesterol esterification and hydrolysis. In this model, efflux values from non-cAMP treated cells are subtracted from efflux to cAMP treated cells to measure ABCA1-dependent efflux.

**In vivo RCT Studies**

**Rationale for choice of LPS dose and route of administration**

Overall, we chose doses and routes of LPS administration based on published literature (mouse and human) 4-10, pilot studies (mouse) and experimental needs (mouse) in order to elicit a reproducible, non-lethal, non-toxic acute inflammatory response. In rodents, we wished to employ a sub-acute model that induced a slower rate of endotoxin release into the blood-stream and therefore eliminated the intravenous (IV) route of administration (ROA). Because we reserve the intraperitoneal (IP) cavity for labeled macrophage administration, we chose the subcutaneous (SQ) route. Given the paucity of published data11 4-7, we performed pilot studies comparing inflammatory and lipid responses across
different LPS administrations, IV LPS (0.1 and 0.3 mg/kg) vs. SQ LPS (3 and 10 mg/kg). All doses produced robust increases in plasma TNFα levels (Supplement figure 3A). Notably, peak inflammatory responses with 3mg/kg SQ were comparable to a much lower IV dose (0.1 mg/kg). FPLC profiles of plasma at 48h revealed a marked induction in LDL-C peak with all LPS doses and ROA with little change in HDL-C mass peak (Supplement figure 3B). We chose 3 mg/kg SQ LPS for our initial studies because this produced a robust, non-toxic (no increase in liver enzymes), acute systemic inflammatory response accompanied by changes in plasma lipoproteins (Supplement figure 1A-F). We chose a lower dose LPS (0.3 mg/kg SQ) in rodents in order to assess the effect of modest hepatic inflammation (~2 fold elevation in plasma levels of hepatic derived SAA) on RCT (especially on the final liver to bile steps) in the setting of low grade systemic inflammation (<3 fold increase in plasma cytokines).

Parenthetically, LPS at 3mg/kg SQ induced ~20 fold peak-increase in plasma cytokines and ~112 fold increase in plasma SAA (Figure 2B) levels in mice, which is of the same order of magnitude as the increases in plasma cytokines (~50 fold) and SAA (~39 fold) (Figure 5) observed with LPS 3ng/kg IV in humans. In contrast, low dose LPS (0.3 mg/kg SQ) in rodents induced ~2-fold increase in plasma cytokines (Figure 4) and ~2 fold increase in plasma SAA.

**Sample Processing for rodent in vivo RCT studies:** Blood, collected via the retro-orbital plexus from anesthetized mice, was centrifuged for plasma supernatant. At 48h mice were sacrificed by cervical dislocation, liver and gall bladder isolated; liver portions (50mg) snap-frozen for lipid and RNA extraction. Tissue lipids were extracted according
to Bligh and Dyer procedure and $^3$H-cholesterol levels in plasma, bile, and extracted lipids measured by liquid scintillation counting (LSC). Feces (0-48h) were collected and soaked overnight in ddH$_2$O (100mg feces/1mL), ethanol added, samples homogenized and free cholesterol isolated by hexane extraction. The aqueous phase was adjusted to pH $\leq$ 1 for bile acid extraction with ethyl acetate. $^3$H-label counts in plasma, liver and feces are expressed as a percentage of total $^3$H-cholesterol injected.

**General Laboratory Methods**

*Lipoprotein Analysis and Enzyme Linked Immunosorbent Assays (ELISA):* Pooled plasma from mice (150µL, N=6) was separated by Fast Protein Liquid Chromatography (FPLC) (Amersham Pharmacia Biotech). Plasma and FPLC fraction total cholesterol levels were measured enzymatically (Wako Pure Chemical Industries). Counts in FPLC fractions (cpm/100µl) were measured by LSC. In humans, following ultracentrifugation, plasma lipoproteins, apolipoproteins, phospholipids and hsCRP were measured enzymatically (Wako Diagnostics, Richmond, VA) on a Hitachi 912 analyzer (Roche Diagnostics, Basel, Switzerland). Levels of murine TNF$\alpha$ and IL6 (R&D systems, Minneapolis, MN) and SAA (Life Diagnostics, West Chester, PA) in plasma and FPLC fractions and human plasma SAA (BioSource, Invitrogen, Eugene, OR) were measured by ELISA.

*RNA isolation and quantitave Real-time PCR analysis:* Liver tissue was homogenized in Trizol using a tissue lyser (Qiagen), RNA (500ng) isolated, and reverse transcribed using an Applied Biosystems High Capacity cDNA archive kit. Levels of mRNAs, using
Applied Biosystems primers, probes and 7300 sequence detector, were assessed by quantitative Real-Time PCR and normalized to β-actin levels.

**Immunoblot analysis:** FPLC fractions were reduced, separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes and probed with apoA-I, apoE and apoB primary antibodies (BIODESIGN International, Meridian Life Science, Maine). Blots were visualized by chemiluminescence (ECL plus kit, GE Healthcare). Liver tissue (50mg) was homogenized in PBS (+protease inhibitors) and centrifuged at low speed (2000xg for 10min). Liver membrane proteins were isolated from supernatant by high-spin ultracentrifugation (56,806xg for 1h at 4°C). The remaining pellet was resuspended in lysis buffer, quantitated by bicinchoninic acid (BCA) assay and equal quantities (10µg/lane) separated by SDS-PAGE electrophoresis prior to transferring to nitrocellulose membranes. Membranes were blocked and probed with ABCA1, ABCG1, SR-BI, ABCG8 and β-actin antibodies (Novus Biologicals, Littleton, CO).
Supplement Tables

Supplement Table 1. Fold change in hepatic mRNA expression of genes at 48h post LPS (3mg/kg, SQ) as assessed by Real-Time PCR (n=6 mice, data presented as mean ± SEM).

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Saline (1.06 ± 0.17)</th>
<th>LPS (3mg/kg) (0.41 ± 0.06)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I</td>
<td>1.06 ± 0.17</td>
<td>0.41 ± 0.06</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>ApoB</td>
<td>1.04 ± 0.15</td>
<td>0.65 ± 0.04</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>ApoE</td>
<td>1.08 ± 0.22</td>
<td>0.41 ± 0.06</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>LDLR</td>
<td>1.01 ± 0.07</td>
<td>0.47 ± 0.04</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>LXR</td>
<td>1.01 ± 0.07</td>
<td>0.78 ± 0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>LCAT</td>
<td>1.01 ± 0.09</td>
<td>0.69 ± 0.02</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>HL</td>
<td>1.05 ± 0.17</td>
<td>0.45 ± 0.08</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>PLTP</td>
<td>1.07 ± 0.18</td>
<td>0.38 ± 0.03</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>PON1</td>
<td>1.06 ± 0.15</td>
<td>0.62 ± 0.04</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

* For comparison of Real-Time data between LPS and saline groups unpaired t-tests were performed.
Supplement Table 2. Endotoxin Reduces HDL Cholesterol Acceptor Function in vivo

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>4 Hours</th>
<th>24 Hours</th>
<th>48 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n=3)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>LPS (3mg/kg) (n=3)</td>
<td>0.61 ± 0.14</td>
<td>0.78 ± 0.13</td>
<td>0.85 ± 0.16</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>P&lt;0.05</td>
<td>P=0.17</td>
<td>P=0.41</td>
</tr>
</tbody>
</table>

*Data representative of 3 independent studies (n=6 mice per treatment group per study).

Plasma from control or LPS treated mice was pooled in each individual study and separated by FPLC. Data presented as mean ± SEM.
Supplement Figures

Supplement Figure 1: Subcutaneous endotoxin (3mg/kg SQ) induces a robust systemic inflammatory response in vivo

Increased plasma protein levels of (A) TNFα and (B) IL6 during endotoxemia (3mg/kg SQ). (C) Induction of hepatic IL6 and TNFα mRNA following LPS. (D) Plasma levels of the liver enzyme, alanine aminotransferase (ALT) were measured enzymatically (Wako Diagnostics, Richmond, VA) on a Hitachi 912 analyzer (Roche Diagnostics, Basel, Switzerland). There was no evidence for increase in ALT at any time point post-LPS (n=3 mice per group). (E) Total cholesterol levels in mice following endotoxin (n=6). (F) FPLC cholesterol profiles at 48h. A marked increase in the LDL-cholesterol peak was observed with no change in HDL-cholesterol mass peak. An increase in cholesterol in the “shoulder” region between LDL and HDL peaks (Fractions 22-28) was also observed. Statistical significance is presented as *p<0.05, **p<0.01 and ***p<0.001 in all figures.

Supplement Figure 2: AcLDL-loaded J774 macrophages express abundant ABCA1 and support robust efflux to ApoA-I

(A) J774 macrophages were treated ± acLDL (20µg/ml) ± cAMP (0.3mM) for 24h prior to harvesting in RIPA buffer. Immunoblot analysis revealed an increase in ABCA1 protein levels with both acLDL (20µg/ml) and cAMP (0.3mM) treatment. (B) J774 macrophages were loaded and labeled for 24h (RPMI, 50µg/ml gentamicin, 1µCi/ml ³H-cholesterol and 50µg/ml acLDL) prior to equilibration for a further 24h (RPMI, 50µg/ml gentamicin and 0.2 % BSA). Cells were washed and efflux to apoA-I (20µg/ml), HDL3
(25µg/ml) or media alone (MEM) was monitored over a 4h efflux period. Loaded J774 macrophages support robust efflux to ApoA-I and HDL3 compared with efflux to MEM (n=4, *p<0.05, **p<0.01 and ***p<0.001).

**Supplement Figure 3: Pilot studies of route and dose of LPS administration**

(A) The effect of different doses of LPS administered SQ and IV on inflammatory markers and lipoprotein changes were compared. All doses increased plasma TNFα levels; however peak effects of 3mg/kg LPS SQ were similar to a much lower IV dose (0.1 mg/kg) (n=4, *p<0.05, **p<0.01 and ***p<0.001). (B) FPLC profiles of plasma at 48h revealed a marked induction in LDL-C peak with all LPS doses and little change in HDL-C mass peak.
References


Supplement Figure 1

A) TNFα (pg/ml)

B) IL6 (pg/ml)

C) Hepatic cytokine (Fold change)

D) ALT (U/L)

E) Total cholesterol (mg/dL)

F) Cholesterol (mg/dL)
Supplement Figure 2

A

acLDL (20µg/ml):
- - + +
cAMP (0.3mM):
- + - +

B

% Efflux/4h

MEM ApoAI HDL3
Acceptor

0.0 0.5 1.0 1.5 2.0 2.5

0.0 0.5 1.0 1.5 2.0 2.5

** ***
Supplement Figure 3

A

![Graph showing TNFα levels in different treatment groups.](image)

- Saline
- 3mg/kg SQ
- 10mg/kg SQ
- 0.1mg/kg IV
- 0.3mg/kg IV

B

![Graph showing cholesterol levels across different treatment groups.](image)

- Control
- IV (0.1mg/kg)
- IV (0.3mg/kg)
- SQ (3mg/kg)
- SQ (10mg/kg)