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Inflammation Impairs Reverse Cholesterol Transport *in vivo*

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Abstract

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

Methods and Results—We employed sub-acute 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 (3mg/kg, SQ) reduced ^{3}H-cholesterol movement from macrophage to plasma and ^{3}H-cholesterol associated with HDL fractions. At 48h bile and fecal counts were markedly reduced consistent with downregulation of hepatic expression of ABCG5, ABCG8 and ABCB11 biliary transporters. Low dose LPS (0.3mg/kg, SQ) 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*, LPS impaired ^{3}H-cholesterol efflux from human macrophages to apoA-I and serum coincident with reduced expression of the cholesterol transporter, ABCA1. During human (3ng/kg; n=20) and murine endotoxemia (3mg/kg, SQ), e*x 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.

Keywords

atherosclerosis; inflammation; cholesterol; lipoproteins; macrophages

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Disclosures

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INTRODUCTION

Reverse cholesterol transport (RCT) is the process by which cholesterol in peripheral cells (e.g., 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 anti-atherogenic function of HDL[3]. Inflammation is proposed to impair HDL function and RCT. This may be of pathophysiological significance as attenuation of RCT might contribute to atherosclerosis in chronic inflammatory states, including metabolic syndrome and Type II diabetes. In this 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.

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 catabolise HDL phospholipids[6–8], 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 SR-BI[10], and reduced expression of hepatic transporters involved in excretion of cholesterol[11] and bile-acids[12]. However, the integrated effect of *in vivo* inflammation on RCT and the relative impact on individual RCT steps has not been examined.

We employed sub-acute 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 ^{3}H-cholesterol from intra-peritoneally injected J774 macrophages onto HDL in plasma and subsequent uptake by liver and clearance into bile and feces. Further, *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.

MATERIALS AND METHODS

Cell Culture

J774 macrophage preparation for RCT and ex vivo cholesterol efflux studies

—RCT studies were performed as previously described[13–16]. J774 macrophages were grown in suspension and incubated for 48h in labeling media containing acetylated-LDL (25μg/mL) and ^{3}H-cholesterol (5μCi/mL). Cells were washed, equilibrated, centrifuged and re-suspended in minimal essential media (MEM) prior to i.p. injection. For murine *ex vivo* efflux studies, J774 macrophages were labeled and loaded identically to *in vivo* studies (supplemental methods). For human *ex vivo* efflux studies, we used a simple, established model of ABCA1 efflux[17, 18]-unloaded J774 macrophages +ACAT inhibitor (2μg/ml) ± cAMP (0.3mM).

In *ex vivo* cellular cholesterol efflux studies, plasma from mice was collected at 0, 6, 24 and 48h post lipopolysaccharide (LPS) (3mg/kg SQ). Serum from humans was collected at 4, 8,

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12 and 24h post LPS (3ng/kg IV). ApoB-containing lipoproteins were removed from plasma/serum by polyethylene glycol (PEG) precipitation[19]. *Ex vivo* efflux from labeled macrophages to 2.8% HDL supernatant or MEM control was measured over 4h. Efflux of ^{3}H-cholesterol 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% FBS, 100ng/mL M-CSF). Macrophages were loaded and labeled for 24h (RPMI, 10% FBS, 50μg/ml acLDL, 6μCi/mL ^{3}H-cholesterol), then washed, equilibrated and treated ± LPS (100ng/ml) for 4h. Cholesterol efflux to human apoA-I (20μg/ml), HDL-3 (50μg/ml), serum (2.5%) and MEM control was assessed over a 4h period. Protein and mRNA expression of cholesterol transporters were also assessed after 4h LPS 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/dose of LPS and pilot study data is presented in supplemental data. Anesthetized mice were injected sub-cutaneously (SQ) with LPS (0.3 or 3mg/kg) or PBS 4h prior to and 24h after intra-peritoneal (IP) injection of labeled macrophages (~4.5 million cpm/2.3 million cells/mouse). Blood was collected, liver and gall bladder isolated, and prepared for lipid, protein and mRNA studies as described previously[13, 14] and in the online supplement. ^{3}H-label counts in plasma, liver and feces are expressed as a percentage of total ^{3}H-cholesterol injected. The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) approved rodent studies and mice were handled according to IACUC guidelines.

Human Endotoxemia Studies

Healthy volunteers (N=20, 50% female, aged 18–40), 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 pre and post intravenous endotoxin infusion of 3ng/kg US standard reference endotoxin (lot # CC-RE-LOT-1+2, Clinical Center, NIH), a dose that we have shown produces a robust inflammatory response[20]. The Penn Institutional Review Board (IRB) approved the study and written informed consent was provided.

Laboratory Methods

A description of general laboratory methods including lipoprotein analysis, quantitative Real-Time PCR and immunoblot analysis are available in supplemental methods.

Statistical analysis

Data are reported as mean ± SEM. For mouse experiments with LPS and saline treatments over multiple time-points, we performed two-way repeated measures analysis of variance (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 LPS and saline

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treated groups at a single time-point (liver, bile, feces, cellular efflux and mRNA data) unpaired t-tests were performed. Endotoxemia effects over time in humans were tested by one-way repeated measures ANOVA and, when significant, post-hoc Bonferroni corrected t-tests were performed comparing time-points post-LPS to baseline. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) and Stata 9.0 software (Stata Corp, College Station, TX) were used for statistical analyses. Statistical significance is presented as *$p<0.05$, **$p<0.01$ and ***$p<0.001$ in all figures.

Statement of Responsibility

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 following endotoxin challenge

Subcutaneous (SQ) administration of LPS (3mg/kg) induced systemic and hepatic inflammation (Supplement figure 1). Plasma levels of TNFα increased transiently while IL6 had more sustained elevation (Supplement figure 1A&B). Hepatic mRNA levels of both cytokines were elevated at 6h (Supplement figure 1C). No evidence of hepatotoxicity, as reflected in plasma alanine aminotransferase levels, was observed during endotoxemia (Supplement figure 1D).

As reported previously[4, 22–24], LPS increased total plasma cholesterol levels (Supplement figure 1E). FPLC profiles (48h) confirmed increased LDL-cholesterol mass with LPS (~2 fold) but minimal effect on HDL-cholesterol mass (Supplement figure 1F). Increased apoB protein in FPLC LDL fractions was observed (Figure 1F&G) and coincided with reduced hepatic expression of the LDL-receptor (Supplement table 1). An increase in cholesterol mass levels in the shoulder region between LDL and HDL peaks was also observed with LPS (1.7 fold increase); these particles were apoA-I/apoB poor and apoE/SAA enriched (Figure 1F&G and Figure 2D). Increased HDL-associated apoE was observed despite reduced hepatic apoE mRNA levels during endotoxemia (Supplement table 1).

Initial RCT experiments were performed with 3mg/kg LPS (Figures 1–3), a dose that induced significant lipoprotein changes as well as systemic and hepatic inflammation. Subsequently, we used a lower dose of LPS (0.3mg/kg; Figure 4), in order 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 LPS (3mg/kg, SQ) on ^{3}H-cholesterol movement from intra-peritoneal macrophages into the plasma compartment was monitored over time. Analysis of pooled data across multiple studies (nine studies, n=6 mice per treatment group per study) showed a moderate but consistent reduction in plasma counts at 4 and 24 h with no difference observed at 48h (Table 1, Figure 1A).

FPLC profiles of plasma from three separate experiments were analyzed to assess the distribution of cholesterol mass and label across lipoproteins during endotoxemia. In control

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mice, the majority of counts (~65%) were found in HDL fractions. At 4h, counts in HDL fractions were reduced by 61% with LPS and remained attenuated at 24h (−44%) and 48h (−16.5%) (Figure 1D&E). In contrast, little change in HDL-cholesterol mass was observed with LPS at 4h (1.17 fold control), 24h (1.07 fold control) and 48h (1.04 fold control) (Figure 1B&C). There was a reduction in the ratio of cholesterol counts/mass, i.e. the rate of enrichment of HDL with new ^{3}H-cholesterol from macrophage following LPS (Supplement table 2). Analysis of FPLC fractions revealed decreased apoA-I and increased apoE and SAA content in HDL fractions following LPS (Figure 1F&G and Figure 2C&D). A marked induction in hepatic SAA mRNA (Figure 2A) coincided with increased plasma protein levels (Figure 2B). In summary, during endotoxemia we found (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 ^{3}H-cholesterol movement from macrophages to HDL.

Endotoxemia impairs murine HDL efflux capacity *ex vivo*

These findings suggest LPS reduced ^{3}H-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 following PEG precipitation of apoB) to promote ^{3}H-cholesterol efflux from loaded J774 macrophages. Efflux from macrophages to HDL supernatant collected at 6, 24 and 48h post-LPS (from a 3mg/kg SQ non-RCT study) was significantly reduced compared to 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 ^{3}H-cholesterol secretion

We assessed the effects of inflammation on later steps in the RCT pathway involving hepatic ^{3}H-cholesterol uptake and flux through liver to bile and feces. Analysis of hepatic counts across multiple studies revealed a non-significant reduction (−13.5 ± 6.2%) in counts at 48h with LPS (Figure 3A & Table 1). 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 ^{3}H-cholesterol secretion from liver to bile and feces (Table 1, Figure 3). At 48h, LPS 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&D). Effects on transporter mRNAs were observed as early as 6h after LPS; 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 (150kDa)[25] protein levels was observed at all time-points consistent with reduced mRNA levels (Figure 3F, 24h blots presented).

A consistent reduction in fecal counts was also observed after LPS (−62.1 ± 5.7%) (Table 1, Figure 3E). LPS had similar effects on fecal free cholesterol (−71.9 ± 4.8%) and bile-acid

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(−65.2 ± 5.9%) levels (pooled data representative of three studies, n=6 per treatment group per study, p<0.001).

Low-dose endotoxin selectively blocks hepatic cholesterol and bile-acid secretion

As the effects of a moderate SQ LPS dose on bile counts were dramatic, we hypothesized that mild "sub-clinical" inflammation with low-dose LPS might attenuate liver to bile ^{3}H-cholesterol even in the absence of significant effects on lipoprotein levels or HDL functionality. Low-dose LPS (0.3mg/kg, SQ) had minimal effects on plasma TNFα 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 48h, n=6, p<0.01), but had no effect on serum lipoprotein FPLC profiles (Figure 4C). Low-dose LPS did not reduce ^{3}H-cholesterol plasma counts (Figure 4B), had no impact on HDL cholesterol counts/mass ratio (1.1) (Figure 4C&D), and did not reduce liver counts (Figure 4E). Nonetheless, low-dose LPS significantly reduced bile (−36%) and fecal counts (−69%) (Figure 4F&G) which correlated with reduced ABCG5 (−23%), ABCG8 (−37%) and ABCB11 (−44%) mRNAs (Figure 4H).

Endotoxemia modulates the reverse cholesterol transport pathway in humans

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

Endotoxin impairs human macrophage efflux function *in vitro*

Inflammation may also directly impair human macrophage cholesterol efflux, the first step of the RCT pathway[9, 26, 27]. LPS (100ng/ml for 4h) reduced *in vitro* ^{3}H-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, 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

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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 LPS, 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 ^{3}H-cholesterol counts at early time-points post LPS with less pronounced effects at later time-points. As 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 due to reduced hepatic clearance of HDL-cholesterol[13]. Indeed, our data suggest that inflammation may reduce the flux of ^{3}H-cholesterol from plasma through liver due to 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 48h plasma counts.

We performed *in vitro* studies examining the direct impact of LPS on macrophage cholesterol efflux using a “foam-cell”model of cholesterol-loaded, primary human monocyte-derived macrophages. LPS 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[28, 29]. Despite reductions in SR-BI mRNA and protein, the lack of effect of LPS 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[28–30]. These findings suggest that inflammation may chronically impair ABCA1-mediated cholesterol efflux from arterial foam-cells, thus accelerating atherosclerosis.

Reduced ^{3}H-cholesterol in plasma may also reflect reduced capacity of inflammatory HDL particles to efficiently accept cholesterol from macrophages. HDL particles undergo remodelling during acute inflammation through activation of lipases such as $sPLA_2$[31] and endothelial lipase (EL)[31, 32], induction of, and HDL association with, serum amyloid A[33], attenuation of LCAT[34] (and CETP activity in humans[35]), as well as oxidant modification of apoA-I[36]. In fact, such changes have been shown to modify HDL efflux[27, 34] and anti-inflammatory functions[37]. In our *in vivo* rodent studies, the marked reduction of HDL ^{3}H-cholesterol/cholesterol mass, suggest 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 remodelling 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[38] and, contrary to our findings, demonstrated increased efflux to HDL from mice over-expressing SAA and acute phase HDL. Differences in cell models (J774 macrophages vs. hepatocytes and CHO cells over-expressing SR-BI) and

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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[39–41], 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 by Berbee and Li *et al*[42, 43], and is likely attributable to reduced clearance via the LDL receptor rather than increased production as we and others[42] find reduced hepatic apoE mRNA levels during endotoxemia. We also found that LPS increased total cholesterol and apoB levels as has been reported in rodents[4, 22–24]. Mechanisms of apoB lipoprotein changes include reduced LDL clearance due to down-regulation of LDL-receptor and upregulation of PCSK9 and increased HMG-CoA reductase activation[4, 22, 23]. 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[4, 44], 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 due to activation of inflammatory HDL lipases, EL and $sPLA_2$[31, 45]. We recently demonstrated reduced HDL phospholipids coincided with induction of EL during human endotoxemia[32]. Despite only modest changes in human HDL-C and apoA-I levels, endotoxin markedly increased plasma and HDL-associated SAA. Further, 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 LPS 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 were observed suggesting 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 (ABCG5 and ABCG8) at both the mRNA and protein level. These changes correlated with reduced bile counts and fecal elimination of ^{3}H-cholesterol, the final step of RCT. In fact, low-dose endotoxin also reduced bile and fecal counts and expression of bile-acid and cholesterol transporters, in the absence of significant systemic inflammation and changes in plasma or hepatic ^{3}H-cholesterol levels. Thus even modest hepatic inflammation may result in selective attenuation of these final steps of RCT.

The endotoxemia model used in our rodent and human studies provides proof of principle that inflammation attenuates RCT while being broadly relevant to human inflammatory pathophysiologies especially infections, sepsis and acute rheumatological disorders. Although sub-acute in nature, the low-dose rodent studies may be of relevance to chronic conditions associated with hepatic inflammation including nonalcoholic steatohepatitis (NASH)[46], hepatic insulin resistance and metabolic syndrome[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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical Impact

Reverse Cholesterol Transport (RCT) is the process by which cholesterol from peripheral cells, such as lipid-laden macrophages in atherosclerotic lesions, is effluxed onto circulating HDL particles to be returned to liver for secretion into bile and feces. Promotion of RCT is considered to be 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 this paper, 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 down-regulating specific biliary transporters, e.g., ABCG5 and ABCG8. This work supports the concept that in chronic inflammatory states, including obesity, metabolic syndrome and type-2 diabetes, impaired RCT may contribute to atherosclerosis and its clinical consequences.

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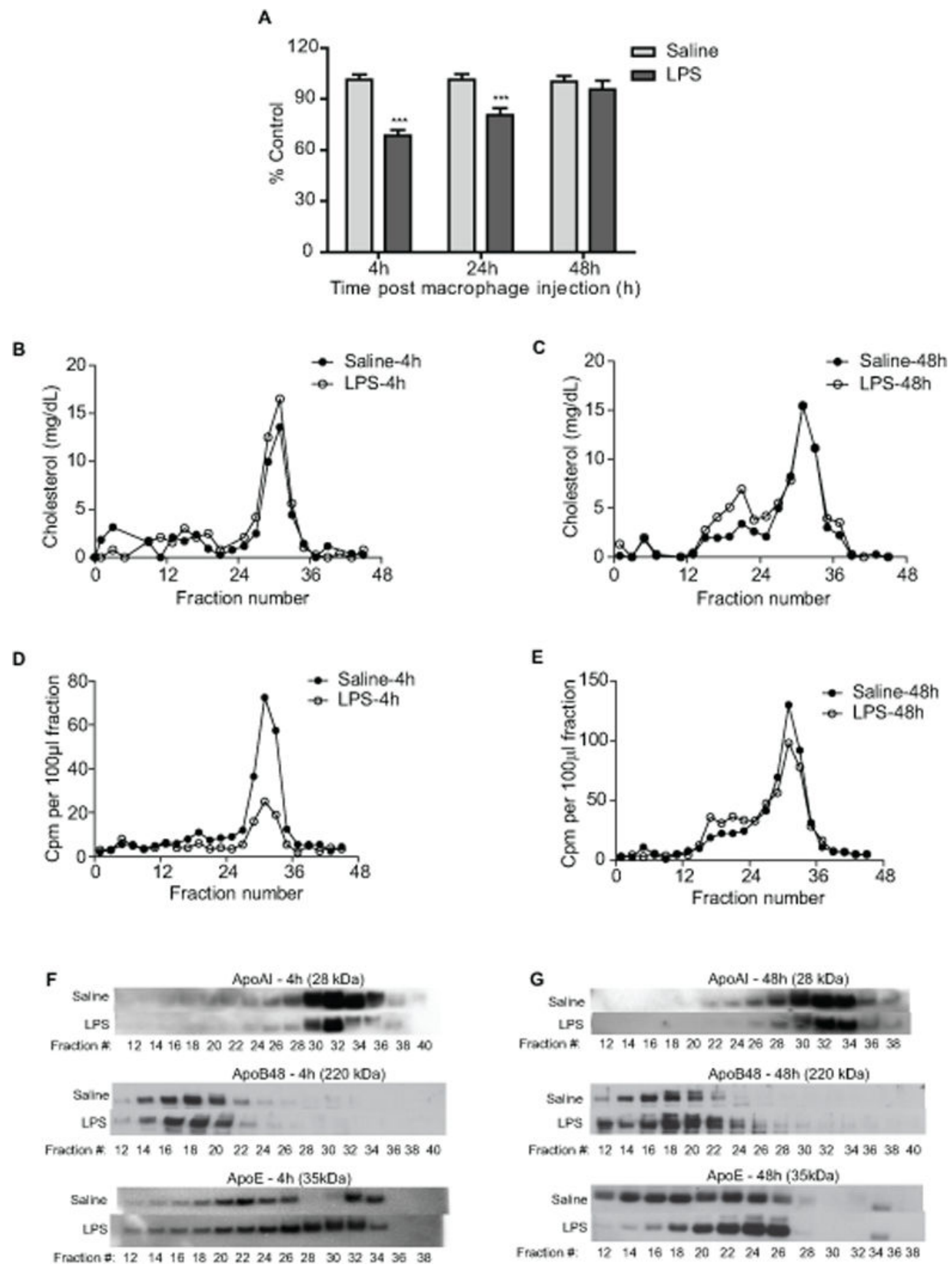

Figure 1. LPS (3mg/kg, SQ) impairs macrophage ^{3}H-cholesterol efflux into plasma *in vivo*

(A) Plasma counts, measured over time across multiple studies, were normalized within each individual study and percentage reduction at indicated time-points post-LPS presented (n=54). A representative FPLC profile (one of three) is presented and revealed (B) no change in cholesterol mass at 4h and (C) increased LDL and no change in HDL-cholesterol mass at 48h after endotoxin. LPS reduced ^{3}H-cholesterol associated with HDL fractions at (D) 4h and (E) 48h. Immunoblot analysis of FPLC fractions revealed a modest reduction in

apoA-I and increase in apoB and apoE levels at (F) 4h and (G) 48h following LPS (3mg/kg) (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

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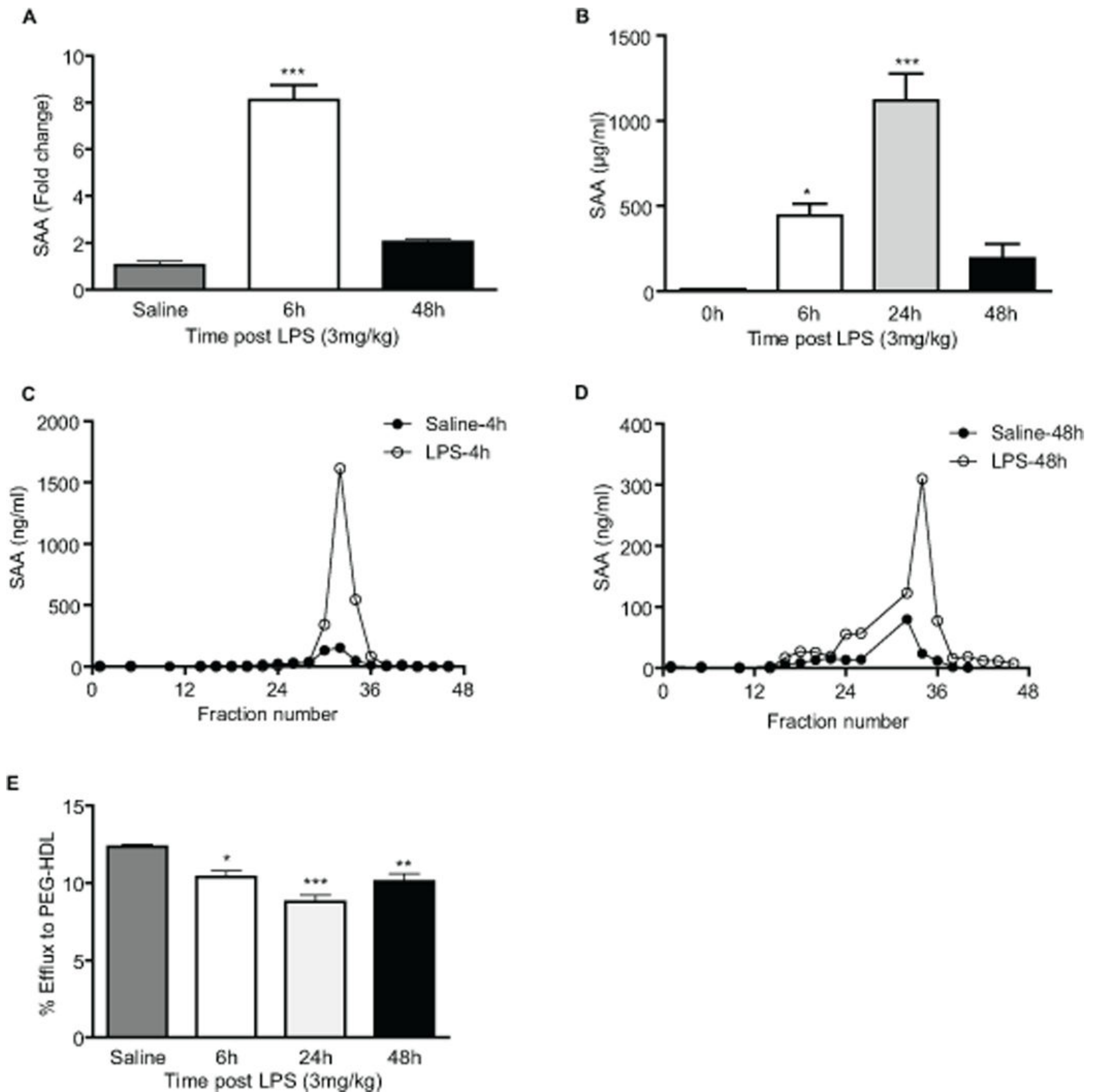

Figure 2. HDL remodeling during endotoxemia impairs HDL acceptor function

(A) Endotoxemia (3mg/kg) induced hepatic serum amyloid A (SAA) mRNA. (B) Plasma SAA protein levels markedly increased following LPS (n=6). FPLC profiling demonstrated the majority of SAA was associated with HDL fractions at (C) 4h and (D) 48h; further SAA was detectable in the apoE rich "shoulder" region at 48h (Fractions 22–28). (E) *Ex vivo* cholesterol efflux from ^{3}H-cholesterol-labeled, acLDL-loaded J774 macrophages to HDL isolated from LPS treated mice (n=5) was reduced at all time-points post-LPS (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

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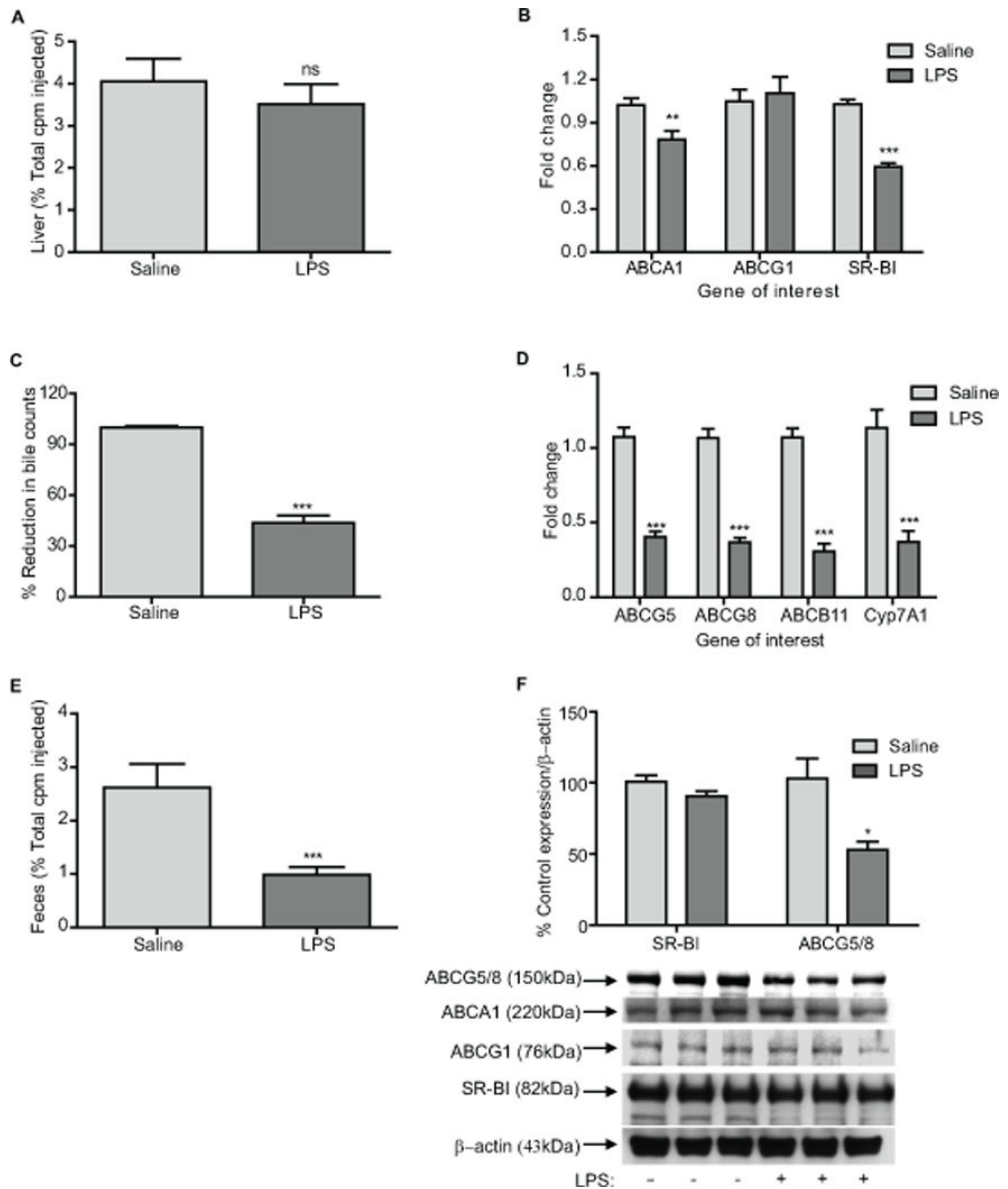

Figure 3. Endotoxin impairs ^{3}H-cholesterol movement through liver to bile and feces

(A) No significant effect on liver counts was observed at 48h following LPS; (B) Reduced mRNA expression of ABCA1 and SR-BI was observed across multiple studies with no effect on ABCG1 expression. (C) LPS markedly reduced bile counts at 48h. This coincided with (D) reduced mRNA expression of hepatic transporters ABCG5, ABCG8 and ABCB11 and the bile-acid synthesis enzyme CYP7A1, as well as (E) reduced ^{3}H-cholesterol levels in feces (n=54). (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 24h. Densitometry data, normalized to β-actin, is shown for SR-BI and ABCG5/8 heterodimer (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

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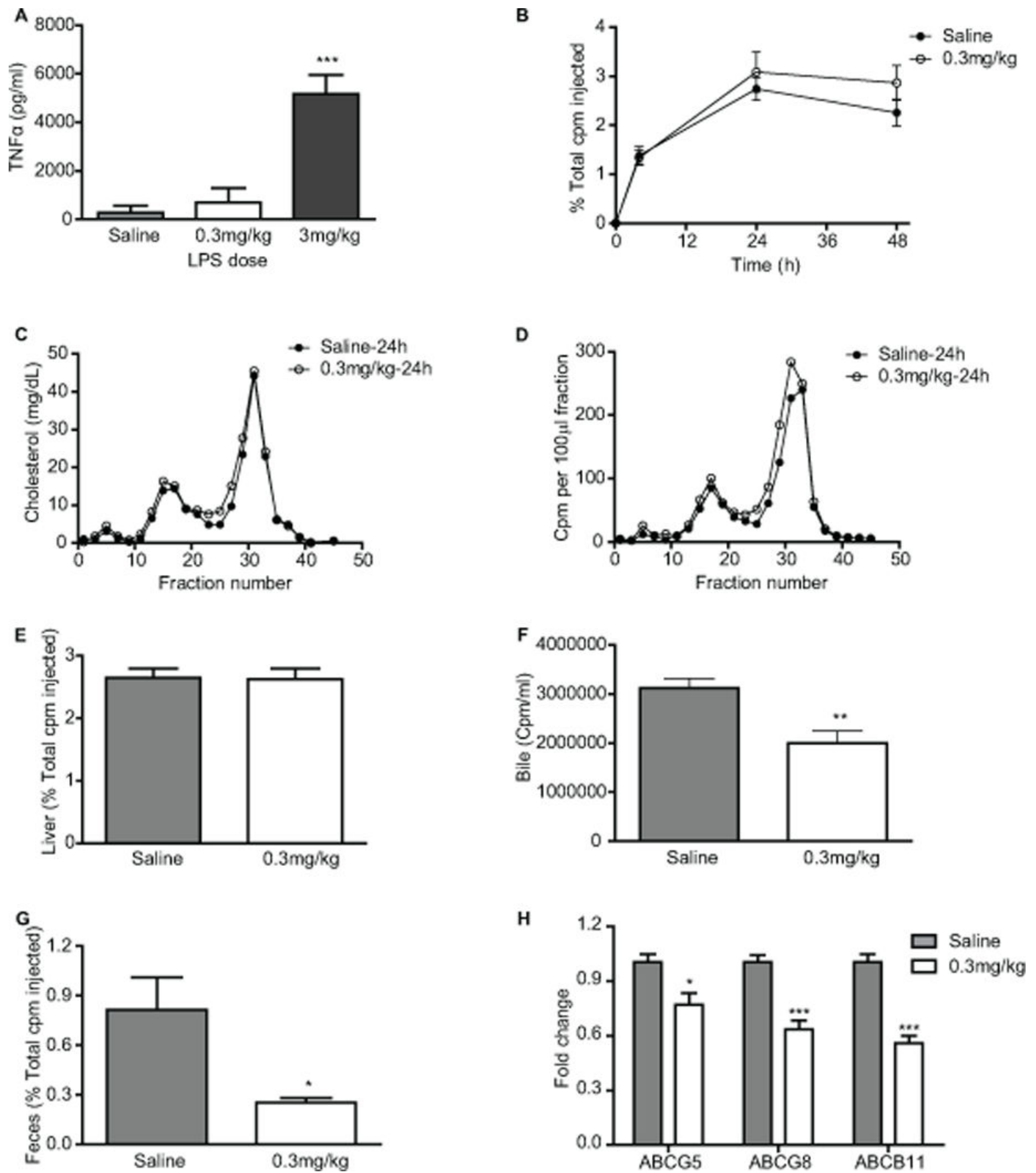

Figure 4. Low-dose endotoxin selectively impairs cholesterol transport through liver to bile and feces

Low-dose endotoxin (0.3mg/kg) (A) induced no significant increase in plasma TNFα protein levels, (B) did not reduce ^{3}H-cholesterol plasma levels and had no effect on (C) FPLC lipid profiles and (D) HDL-associated counts at 24h. (E) ^{3}H-cholesterol tracer counts in liver were not affected. However, low-dose LPS reduced (F) bile and (G) fecal counts as well as (H) hepatic ABCG5, ABCG8 and ABCB11 mRNA expression (n=6, *p<0.05, **p<0.01, ***p<0.001).

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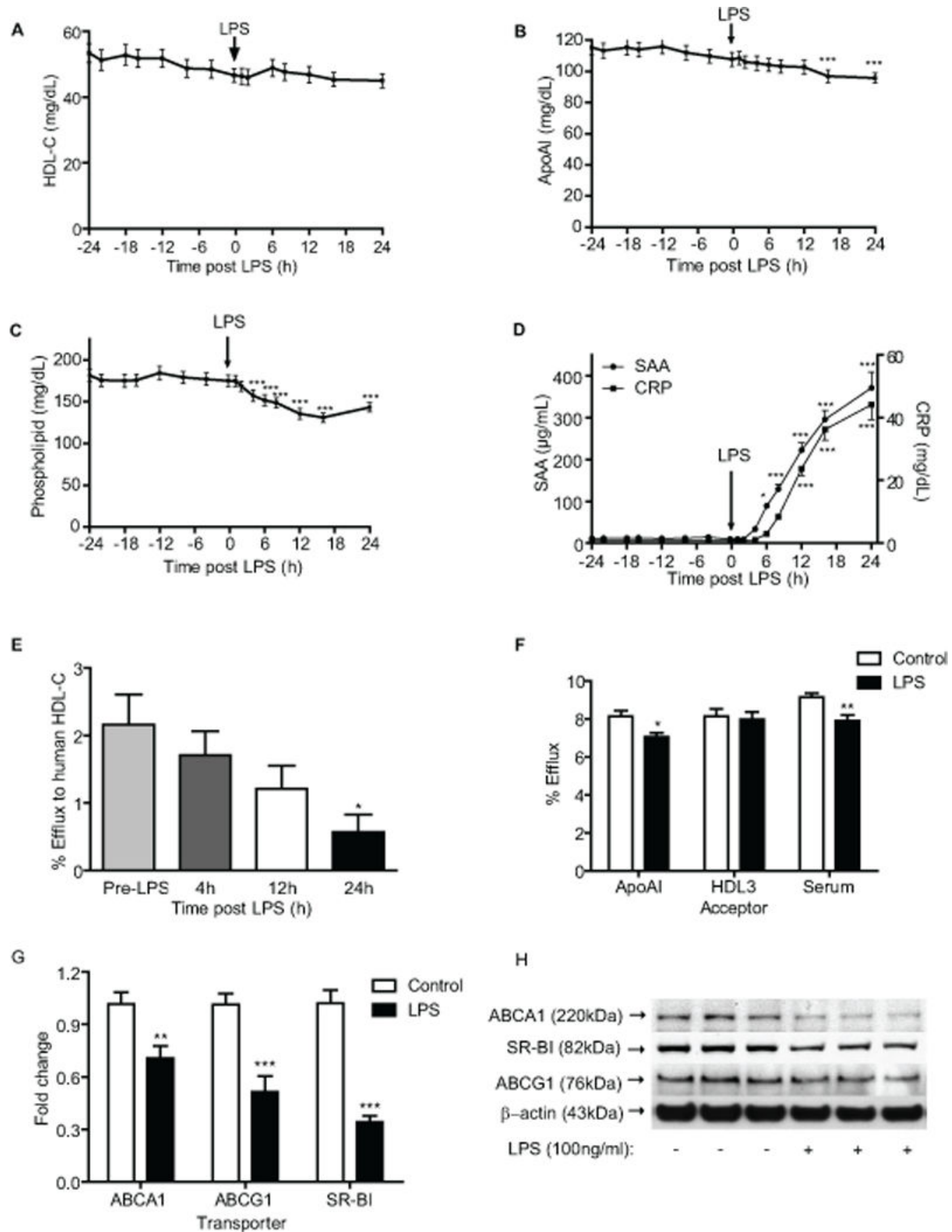

Figure 5. Human acute phase HDL has reduced acceptor capacity for macrophage ^{3}H-cholesterol *ex vivo*

Human experimental endotoxemia (3ng/kg, IV bolus) had non-significant effects on plasma levels of (A) HDL-cholesterol (F=0.39, p=0.91) and moderately decreased (B) ApoA-I at later time-points. (C) A reduction in plasma phospholipid and (D) a marked induction of plasma SAA levels and hsCRP was observed as early as 6h post LPS (n=20). (E) *Ex vivo* ^{3}H-cholesterol efflux from J774 macrophages to acute phase HDL (2.8% PEG supernatant) was reduced at 24h post-LPS (n=20). (F) ^{3}H-cholesterol efflux from LPS treated (100ng/ml for 4h) human macrophages *in vitro* was reduced to apoA-I (20μg/ml) and serum (2.5%) but

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not to HDL3 (50μg/ml). (G) LPS reduced mRNA expression of ABCA1, ABCG1 and SR-BI in human macrophages (cells derived from three people); (H) Immunoblot analysis revealed a marked reduction in ABCA1, a moderate reduction in SR-BI and little change in ABCG1 protein levels (Figure 5H) (*p<0.05, **p<0.01, ***p<0.001).

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Table 1

Effect of LPS (3mg/kg) on Reverse Cholesterol Transport (RCT)

Percent change in ^{3}H-cholesterol counts during endotoxemia compared to saline control (pooled data from nine experiments with six mice per group, n=54 mice per group)				
Counts		**Percent change**	**ANOVA F statistic of Endotoxin Effect**	**P value (compared to saline control)**
Plasma	**4 hours**	↓ 31.6 ± 3.5 %	F = 24.33	p<0.001
	24 hours	↓ 19.5 ± 4 %		p<0.001
	48 hours	↓ 4.4 ± 5.3 %		p=0.96
Liver	**48 hours**	↓13.5 ± 6.2 %	N/A	p=0.47
Bile	**48 hours**	↓56.3 ± 4.5 %	N/A	p<0.001
Feces	**0–48 hours**	↓62.1 ± 5.7 %	N/A	p<0.005

[*] Data are reported as mean ± SEM. For mouse experiments with LPS and saline treatments over multiple time-points, we performed two-way repeated measures analysis of variance (ANOVA) to test for differences in means; when significant post-hoc Bonferroni corrected t-tests were applied. For comparison of data between LPS and saline groups at a single time-point (liver, bile and feces data) unpaired t-tests were performed.