Myeloperoxidase and serum amyloid A contribute to impaired in vivo reverse cholesterol transport during the acute phase response but not group IIA secretory phospholipase A_2 [S]

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Abstract Atherosclerosis is linked to inflammation. HDL protects against atherosclerotic cardiovascular disease, mainly by mediating cholesterol efflux and reverse cholesterol transport (RCT). The present study aimed to test the impact of acute inflammation as well as selected acute phase proteins on RCT with a macrophage-to-feces in vivo RCT assay using intraperitoneal administration of [^{3}H]cholesterol-labeled macrophage foam cells. In patients with acute sepsis, cholesterol efflux toward plasma and HDL were significantly decreased ($P < 0.001$). In mice, acute inflammation (75 μg/mouse lipopolysaccharide) decreased [^{3}H] cholesterol appearance in plasma ($P < 0.05$) and tracer excretion into feces both within bile acids (−84%) and neutral sterols (−79%, each $P < 0.001$). In the absence of systemic inflammation, overexpression of serum amyloid A (SAA, adenovirus) reduced overall RCT ($P < 0.05$), whereas secretory phospholipase A_2 (sPLA$_2$, transgenic mice) had no effect. Myeloperoxidase injection reduced tracer appearance in plasma ($P < 0.05$) as well as RCT (−36%, $P < 0.05$). Hepatic expression of bile acid synthesis genes ($P < 0.01$) and transporters mediating biliary sterol excretion ($P < 0.01$) was decreased by inflammation. In conclusion, our data demonstrate that acute inflammation impairs cholesterol efflux in patients and macrophage-to-feces RCT in vivo in mice. Myeloperoxidase and SAA contribute to a certain extent to reduced RCT during inflammation but not sPLA$_2$. However, reduced bile acid formation and decreased biliary sterol excretion might represent major contributing factors to decreased RCT in inflammation.—Annema, W., N. Nijstad, M. Tölle, J. F. de Boer, R. V. C. Buijs, P. Heeringa, M. van der Giet, and U. J. F. Tietge. **Myeloperoxidase and serum amyloid A contribute to impaired in vivo reverse cholesterol transport during the acute phase response but not group IIA secretory phospholipase A_2. *J. Lipid Res.* 2010.** 51: **743–754.**

Supplementary key words feces • inflammation • sepsis • atherosclerosis • mice

Epidemiological studies established a strong inverse association between plasma HDL cholesterol levels and the risk of atherosclerotic cardiovascular disease (CVD) (1, 2). A major anti-atherogenic activity of HDL is regarded to be reverse cholesterol transport (RCT), a process comprising removal of excess cholesterol from peripheral cells, most importantly macrophage foam cells in atherosclerotic lesions, and transport back to the liver for subsequent excretion into bile and feces (2, 3). Understanding the pathophysiological factors regulating RCT is therefore of prime importance.

Inflammation is strongly linked to atherosclerosis (4–6). The atherosclerotic plaque itself is increasingly considered a site of chronic inflammation within the vessel wall (5, 7). In addition, markers of inflammation are elevated in plasma of patients with established atherosclerotic CVD, and circulating levels of several acute phase proteins

Abbreviations: apo, apolipoprotein; APP, acute phase protein; APR, acute phase response; CVD, cardiovascular disease; FPLC, fast protein liquid chromatography; sPLA$_2$, group IIA secretory phospholipase A_2; ICU, intensive care unit; LPS, lipopolysaccharide; MPO, myeloperoxidase; RCT, reverse cholesterol transport; SAA, serum amyloid A; SR-BI, scavenger receptor BI.

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(APPs) have been shown to possess a predictive value for future cardiovascular events (8, 9). Recently, it was also demonstrated that eliminating a large part of the hepatic acute phase response (APR) by knocking out the gp130 receptor on hepatocytes confers significant protection from atherosclerosis, indicating that APP might mechanistically contribute to the atherosclerotic process (10). Furthermore, inflammation impacts on HDL metabolism resulting in accelerated HDL catabolism and substantial remodeling of the HDL particle (11–13), making it likely that these changes might translate into altered in vivo RCT.

Therefore, the aim of the present study was to assess whether induction of the APR affects in vivo RCT and to dissect out the role of important APP that have been established to regulate HDL metabolism during an inflammatory response. Our data demonstrate that acute inflammation transiently reduces cholesterol efflux to plasma in patients with an APR and impairs macrophage-to-feces RCT in vivo in mice. Group IIA secretory phospholipase A_2 ($sPLA_2$) and myeloperoxidase (MPO), but not serum amyloid A (SAA), contribute to reduced cholesterol efflux toward plasma. However, although expression of $sPLA_2$ in the absence of generalized inflammation did not affect macrophage-to-feces RCT, SAA overexpression and MPO infusion decreased in vivo RCT significantly.

MATERIALS AND METHODS

Patients

Consecutive patients admitted with acute sepsis to the intensive care unit (ICU) at Charité–Campus Benjamin Franklin in Berlin, Germany were included if they met established criteria for septic shock (14). Patients under 18 years of age, pregnant women, and patients with preexisting endocrine disorders were excluded. A first blood sample was obtained during a routine blood draw at admittance (d0) and a second blood sample was drawn at day 21 following complete clinical recovery, defined as SAPSII score below 35, no clinical signs of systemic infection, and no necessity for treatment at the ICU. Blood was immediately placed on ice and plasma was obtained and stored at −80°C until further analysis. Ten patients were included, eight matched healthy volunteers served as controls (for clinical details, see Table 1). The origin of infection in the patients was lungs (n = 5), urinary tract (n = 4), and gallbladder (n = 1). Six patients had gram-negative and 2 gram-positive infection; no bacteria could be isolated in two patients. Organ dysfunction included respiratory (n = 8), cardiovascular (n = 7), renal (n = 4), central nervous system (n = 3), liver (n = 1), and hematologic (n = 1) failure. All patients were receiving inotropic drugs or vasopressors and required mechanical ventilation and hydrocortisone during the acute phase of sepsis. The study was approved by the local Ethics Committee and written informed consent for blood sampling was obtained from patients or their proxies.

Animals

C57BL/6J control mice were purchased from Charles River (Sulzfeld, Germany). The human group IIA $sPLA_2$-transgenic mice expressing the human transgene under the control of the endogenous human promoter have been described previously (15). The $sPLA_2$-transgenic line has been backcrossed to the C57BL/6J genetic background lacking the endogenous murine $sPLA_2$ enzyme due to a frameshift mutation for >14 generations. The animals were caged in animal rooms with alternating 12 h periods of light (from 7 AM to 7 PM) and dark (from 7 PM to 7 AM) with ad libitum access to water and mouse chow diet (Arie Blok, Woerden, The Netherlands). Animal experiments were performed in conformity with Public Health Service policy and in accordance with the national laws. All protocols were approved by the responsible ethics committees of the University of Groningen and the Landesamt für Gesundheit, Ernährung und technische Sicherheit Berlin (LAGETSI).

Construction of recombinant adenoviruses

The human SAA1 cDNA was amplified from IL-6-treated HepG2 cells (ATCC via LGC Promochem, Teddington, UK) by PCR using specific primers according to the human sequence (NM_000331, GenBank) and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). Recombinant adenovirus (AdhSAA) was generated using the Adeno-X kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. The mouse CE/J SAA expressing adenovirus was kindly provided by Dr. Nancy Webb, University of Kentucky, Lexington, KY. An empty adenovirus (AdNull) was used as control (16). Recombinant adenoviruses were amplified and purified as described previously (17). For mouse experiments, a dose of 1 × 10E11 particles/mouse of each of the respective adenoviruses was used.

In vivo reverse cholesterol transport studies

Wild-type C57BL/6J donor mice were injected intraperitoneally with 0.5 ml of 10% Brewer thioglycollate medium (Becton Dickinson, Le Point de Claix, France). On day 4 after thioglycollate injection, peritoneal macrophages were harvested as described (18). Macrophages were plated in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (HyClone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen) and were allowed to adhere for 4 h at 37°C under 5% CO_2 humidified air. Then nonadherent cells were removed by washing twice with PBS followed by loading of the macrophages with 50 μg/ml acetylated LDL and 3 μCi/ml [^{3}H]cholesterol (Perkin Elmer, Boston, MA) for 24 h. After washing twice with PBS, the macrophages were equilibrated for 18 h in RPMI 1640 medium containing 1% penicillin/streptomycin and 2% BSA (Sigma, St. Louis, MO). Immediately before injection, cells were harvested and resuspended in RPMI 1640 medium. As general procedure for in vivo macrophage-to-feces RCT studies, [^{3}H]cholesterol-loaded macrophage foam cells were injected intraperitoneally into individually housed recipient mice. Plasma was collected at the indicated time points after macophage injection by retroorbital puncture and for the final blood draw by heart puncture. At the end of the experimental period, livers were harvested, snap-frozen in liquid nitrogen, and stored at −80°C until further use. Feces were collected continuously as indicated up to 48 h. Counts in plasma were assessed directly by liquid scintillation counting (Packard 1600CA Tri-Carb, Packard, Meriden, CT). To determine uptake of tracer into the liver and secretion into the feces, respective samples were processed as indicated below before scintillation counting. All obtained counts were expressed relative to the administered tracer dose.

Specifically, to study the effects of the APR on RCT, C57BL/6J mice were injected intraperitoneally with 75 μg lipopolysaccharide (LPS, *Escherichia coli* O111:B4, Sigma) in PBS, while controls received PBS injections shortly before receiving the labeled macrophage foam cells. LPS injection reduced food intake by 28% and feces production by 34%. For the subsequent experiment as-

sessing the impact of SAA on macrophage-to-feces RCT, C57BL/6J mice were injected with labeled macrophage foam cells on day 3 after receiving AdSAA or the control adenovirus AdNull. To investigate the role of $sPLA_2$ in RCT, $sPLA_2$-transgenic mice were compared with C57BL/6J controls. To assess the impact of MPO on RCT, C57BL/6J mice received intravenously three consecutive injections in 6 h intervals with either PBS (controls) or 50 μg/mouse purified MPO in 200 μl of 1 mM H_2O_2 (19).

Plasma lipid and lipoprotein analysis

Plasma total cholesterol and triglycerides were measured enzymatically using commercially available reagents (Wako Pure Chemical Industries, Neuss, Germany). To determine plasma HDL cholesterol levels, apolipoprotein (apo)B-containing lipoproteins were precipitated using 0.36% phosphotungstic acid (Sigma) and cholesterol content in the supernatant was determined as described above. HDL for efflux and cellular cholesterol uptake studies described below was isolated from patient plasma obtained at d0 and d21 by sequential ultracentrifugation ($1.063 < d < 1.21$) as described previously (15). Pooled plasma samples from mice of the same experimental group were subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Healthcare, Uppsala, Sweden) as described (20). Samples were chromatographed at a flow rate of 0.5 ml/min, and fractions of 500 μl each were collected. Individual fractions were assayed for cholesterol concentrations as described above.

Determination of counts recovered within liver and feces

Counts within liver were determined following solubilization of the tissue (Solvable, Packard) exactly as previously reported (15). Counts recovered from a respective piece of liver were backcalculated to total liver mass. Fecal samples were dried, weighed, and thoroughly ground. Aliquots were separated into bile acid and neutral sterol fractions as previously published (16). Briefly, samples were first heated for 2 h at 80°C in alkaline methanol and then extracted three times with petroleum ether. In the top layer, counts within the neutral sterol fraction were determined by liquid scintillation counting, while counts incorporated into bile acids were assessed from the bottom layer. Counts recovered from the respective aliquots were related to the total amount of feces produced over the whole experimental period.

In vitro efflux assays

Two sets of experiments were performed using either THP-1 cells or primary mouse peritoneal macrophages (see above). THP-1 human monocytes (ATCC via LGC Promochem) were grown in suspension culture in RPMI 1640 medium supplemented with 10% FBS, 50 μM β-mercaptoethanol, and 1% penicillin/streptomycin until differentiation into macrophages by the addition of 100 ng/ml phorbol myristate acetate (PMA, Sigma). Differentiated THP-1 macrophages were loaded with 50 μg/ml acetylated LDL and 3 μCi/ml [^{3}H]cholesterol for 24 h followed by equilibration for 18 h as detailed above for peritoneal macrophages. Then cells were washed with PBS, and either 1% serum from patients and controls (THP-1 macrophages) or 50 μg protein/ml of isolated HDL (peritoneal macrophages) diluted in RPMI 1640 was added. After 24 h, the supernatant was taken off and radioactivity within the medium was determined by liquid scintillation counting. The cell layer was washed twice with PBS, then 0.1 M NaOH was added, plates were incubated for 30 min at room temperature, and the radioactivity remaining within the cells was assessed by liquid scintillation counting. Wells incubated with RPMI but without added serum or HDL were used as blanks to determine efflux not due to serum or HDL addition, and these values were subtracted from the respective experimental values. Efflux is given as the percentage of counts recovered from the medium in relation to the total counts present on the plate (sum of medium and cells).

In vitro cholesterol uptake assays

HDL isolated from patient plasma by sequential ultracentrifugation ($1.063 < d < 1.21$) was labeled with the nonhydrolyzable trap label [^{3}H]cholesteryl ether (Perkin Elmer) essentially as previously described (15). Cholesteryl ether behaves metabolically as cholesteryl ester (20); however, by use of the ether bond resecretion by the cells is prevented. HepG2 cells were used as an established model for human hepatocytes and were cultured as detailed above except that 24 h before adding the labeled HDL preparations (50 μg HDL cholesterol/ml), 10% FBS was substituted by 10% lipoprotein-depleted serum. Labeled HDL preparations were then added to the cells in serum-free DMEM and incubations were continued for 6 h. Supernatants and cells were processed as detailed above for macrophages, and radioactivity within medium and cells was determined. Cellular uptake is expressed as the percentage of counts recovered from the cells in relation to the total counts present on the plate (sum of medium and cells). Additional experiments were performed, in which HepG2 cells were treated for 24 h with a mixture of tumor necrosis factor-α (25 ng/ml), interleukin-1β (10 ng/ml) and interleukin-6 (500 pg/ml) (all from R and D Systems, Minneapolis, MN) and tracer uptake from healthy control HDL was determined essentially as outlined above.

Western blotting

Expression of human SAA was checked by Western blot performed on plasma. Equal amounts of plasma were resolved by SDS-PAGE electrophoresis and blotted onto nitrocellulose (GE Healthcare Biosciences Corp., Piscataway, NJ). SAA was visualized using a commercially available monoclonal mouse anti-human SAA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by the appropriate HRP-conjugated secondary antibody (GE Healthcare, Chalfont St Giles, UK). Western blots for different hepatic transport proteins involved in the biliary secretion process were carried out on liver plasma membranes prepared essentially as described (21). Protein concentrations were determined with the bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of protein were resolved by SDS-PAGE electrophoresis and blotted onto nitrocellulose. Abcg5 was visualized using a rabbit anti-mouse Abcg5 antibody (kindly provided by Dr. Albert Groen, University Medical Center Groningen, The Netherlands) and Abcb11 (Bsep) was detected using a rabbit anti-rat Abcb11 antibody (a kind gift from Dr Bruno Stieger, University Hospital Zurich, Switzerland), followed by the appropriate HRP-conjugated secondary antibody (GE Healthcare). HRP was detected using chemiluminescence (ECL, GE Healthcare). Blots were quantitated using the freely available ImageJ software adjusting the background for the area size of each band and subtracting it from the respective bands. Results were normalized for the average of the control mice.

Determination of plasma SAA, $sPLA_2$, and MPO levels

Commercially available ELISA kits were used according to the manufacturer's instructions to respectively determine human SAA (Biosupply, Bradford, UK), mouse SAA (BioSource, Camarillo, CA), $sPLA_2$ (Cayman Chemicals, Ann Arbor, MI), mouse MPO (Hycult Biotechnology b.v., Uden, The Netherlands), and human MPO (R and D Systems) levels in plasma. Please note that although plasma MPO levels obtained with the human and

mouse MPO-specific systems are comparable, this is not given for the mouse versus the human SAA-specific ELISA systems.

MPO purification

Human MPO was isolated as described previously (22). Briefly, isolated human neutrophils were dissolved in cetylmethyl ammonium bromide (Sigma) and sonicated. The extract was absorbed to a concanavalin A sepharose gel (Pharmacia, Uppsala, Sweden) and eluted with α-methyl-D-mannoside (Sigma). The eluate was further purified on a Sephadex G100 gel (Pharmacia). The final human MPO preparation had an optical density ratio 428/280 of 0.78 and was not contaminated with proteinase 3, elastase or lactoferrin as determined by antigen specific ELISAs. Analysis by gel electrophoresis showed specific bands for human MPO at 15, 38, and 58 kDa.

Analysis of gene expression by real-time quantitative PCR

Total RNA from mouse livers was isolated using Trizol (Invitrogen) and quantified with a NanoDrop ND-100 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA synthesis was performed from 1 μg of total RNA. Real-time quantitative PCR was carried out on an ABI-Prism 7700 (Applied Biosystems, Foster City, CA) sequence detector with the default settings (21). PCR primers and fluorogenic probes were designed with the Primer Express Software (Applied Biosystems) and synthesized by Eurogentec (Seraing, Belgium). The mRNA expression levels presented were calculated relative to the average of the housekeeping gene cyclophilin and further normalized to the relative expression levels of the respective controls.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL). Values are expressed as means ± SEM. Paired or unpaired Student's *t*-test was used as appropriate to assess statistical differences between groups. Statistical significance for all comparisons was assigned at $P < 0.05$.

RESULTS

Acute inflammation impairs macrophage cholesterol efflux to plasma and HDL in sepsis patients

To assess the impact of acute inflammation in human patients on cholesterol efflux from macrophage foam cells, the first step in RCT, in vitro efflux experiments were performed. Blood samples were collected from sepsis patients on the day of admittance to the ICU (d0) and on day 21 following clinical recovery. Cholesterol efflux toward plasma was reduced by 73% in sepsis patients compared with matched healthy controls ($P < 0.001$, **Fig. 1A**). After recovery, cholesterol efflux in the same patients improved significantly ($P < 0.001$) but was still lower than in controls ($P < 0.05$, Fig. 1A). Because plasma HDL cholesterol levels are considerably low in sepsis patients (**Table 1**), we next isolated HDL from patients and controls to delineate whether reduced efflux toward plasma might reflect decreased HDL levels or is due to altered acceptor properties of the HDL particle. Cholesterol efflux toward isolated HDL was also markedly impaired by acute sepsis compared with HDL from the same patients after recovery ($P < 0.001$, Fig. 1B). Next, plasma levels of several acute phase proteins related to HDL metabolism were measured. Plasma SAA ($P < 0.001$), $sPLA_2$ ($P < 0.001$), and MPO ($P < 0.05$) were significantly higher in sepsis patients than in controls (Table 1). After recovery from sepsis, plasma levels of $sPLA_2$ and MPO returned to control values, whereas plasma levels of SAA at day 21 remained still slightly elevated in patients compared with controls ($P < 0.05$, Table 1).

Acute inflammation decreases macrophage-to-feces RCT in vivo in mice

Next we aimed to determine whether an acute inflammatory response would also translate into reduced RCT in vivo, using an established macrophage-to-feces RCT assay (23). LPS injection increased plasma levels of SAA (24 h: 3685 ± 133, 48 h: 40 ± 3 μg/ml vs. 15 ± 2 μg/ml in the PBS controls) as well as MPO (48 h: 67 ± 4 vs. 145 ± 20 ng/ml, $P < 0.01$). Consistent with previous results (24), induction of the APR in mice did not result in significant changes in plasma total cholesterol (102 ± 7 vs. 112 ± 8 mg/dl, n.s.), HDL cholesterol (76 ± 3 vs. 75 ± 10 mg/dl, n.s.) and non-HDL cholesterol (26 ± 3 vs. 37 ± 12 mg/dl, n.s.) levels 48 h after LPS injection, reflected by only minor shifts in the plasma lipoprotein profile as determined by FPLC analysis (**Fig. 2A**). On the other hand, plasma triglyceride levels increased significantly after LPS injection (40 ± 2 vs. 145 ± 36 mg/dl, $P < 0.05$). A single dose of LPS significantly decreased the movement of [^{3}H]cholesterol toward plasma by 33% at 6 h ($P < 0.05$, Fig. 2B) and by 27% at 24 h after injection ($P < 0.05$), whereas counts within plasma were not different at 48 h compared with saline controls. [^{3}H] cholesterol tracer within liver was similar in both experimental groups (Fig. 2C). In contrast, tracer recovery within feces was markedly reduced by LPS injection, both within the bile acid ($P < 0.001$) and the neutral sterol fraction ($P < 0.001$, Fig. 2D). These data demonstrate, consistent with a previous report (25) that acute inflammation results in impaired RCT from macrophages to feces in vivo.

Overexpression of human and mouse SAA has divergent effects on macrophage-to-feces RCT in vivo

SAA is an APP with apolipoprotein properties that preferentially associates with HDL (13, 24, 26). To investigate whether SAA contributes to decreased RCT during the APR, human and mouse SAA were specifically overexpressed in the absence of generalized inflammation using recombinant adenoviruses. Human SAA overexpression did not impact on the plasma lipoprotein profile as revealed by FPLC analysis and also left plasma [^{3}H]cholesterol counts, liver counts, and tracer excretion into the feces essentially unchanged (supplementary Fig. I). Plasma levels of human SAA following recombinant adenovirus administration were 1020 ± 284 μg/dl at the 48 h time point. In contrast, mouse SAA overexpression increased LDL cholesterol in plasma (**Fig. 3A**). Although plasma (Fig. 3B) and liver counts (Fig. 3C) were not significantly affected, fecal [^{3}H]cholesterol tracer excretion was significantly decreased compared with control adenovirus-injected mice, indicating lower overall RCT in response to SAA overexpression ($P < 0.05$, Fig. 3D). Plasma mouse SAA

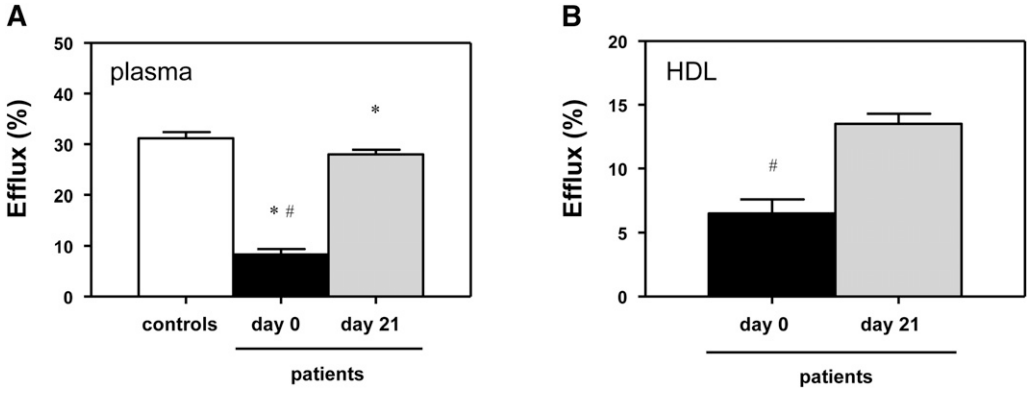

Fig. 1. Acute inflammation decreases macrophage cholesterol efflux in vitro toward plasma and HDL from sepsis patients. A: Efflux from THP-1 macrophages loaded with [^{3}H]labeled cholesterol and acetylated LDL toward 1% plasma for 24 h. B: Efflux (24 h) from C57BL/6J thioglycollate-elicited peritoneal macrophages loaded with [^{3}H]labeled cholesterol and acetylated LDL toward HDL (50 μg/ml) isolated by sequential ultracentrifugation. Experiments were performed as described under Materials and Methods. Data are given as means ± SEM. n = 10 for patients and n = 8 for controls. * indicates statistically significant differences from healthy controls (at least $P < 0.05$), and # indicates statistically significant differences from patients at day 21 ($P < 0.001$) as assessed by Student's *t*-test. Day 0, patients with acute sepsis at the day of admittance to the ICU; day 21, the same patients following full recovery from sepsis 21 days after admittance.

levels in response to AdmSAA injection were 404 ± 96 μg/ml at the 48 h time point and 4.1-fold higher at the start of the experiment (0 h).

sPLA$_2$ overexpression reduces cholesterol efflux toward plasma, but does not affect macrophage-to-feces RCT in vivo

Similar to SAA, plasma sPLA$_2$ levels are strongly elevated during human inflammation and sPLA$_2$ has been shown to increase HDL catabolism in transgenic mice (15, 27). To explore the effects of sPLA$_2$ on RCT, we used transgenic mice specifically overexpressing human sPLA$_2$ in the absence of systemic inflammation. These mice had on average 4.7-fold higher plasma sPLA$_2$ levels compared with the septic patients, and were on the C57BL/6J genetic background that lacks endogenous expression of the murine enzyme due to a frameshift mutation (15). Consistent with previously published data (15), HDL cholesterol levels were lower in sPLA$_2$ transgenic mice as revealed by FPLC analysis (**Fig. 4A**). sPLA$_2$ overexpression decreased the appearance of [^{3}H]cholesterol in plasma 6 h ($P < 0.05$, Fig. 4B), 24 h ($P < 0.05$) and 48 h ($P < 0.05$) after macrophage injection. There was no significant difference in the recovery of [^{3}H]cholesterol within liver between sPLA$_2$ transgenic mice and C57BL/6J controls (Fig. 4C). Fecal tracer excretion within the bile acid fraction was lower in sPLA$_2$ overexpressing mice ($P < 0.05$, Fig. 4D), whereas tracer excretion within the neutral sterol fraction and total RCT remained essentially unchanged. These results indicate that sPLA$_2$ expression might contribute to decreased cholesterol efflux in response to acute inflammation; however, sPLA$_2$ has no effect on overall macrophage-to-feces RCT during the APR.

TABLE 1. Clinical characterization of patients and controls

	Patients n = 10		Controls n = 8
Male/female	6/4		5/3
Age (years)	59 ± 4		53 ± 2
BMI (kg/m^2)	25.4 ± 0.9		23.5 ± 1.0
	d 0	d 21	
SAPSII score	63 ± 2	31 ± 1*	n.d.
Total cholesterol (mg/dl)	109 ± 5	187 ± 9*	163 ± 9*
HDL cholesterol (mg/dl)	25 ± 2	48 ± 3*	47 ± 4*
Triglycerides (mg/dl)	139 ± 6	254 ± 12*	133 ± 11‡
SAA (μg/dl)	823 ± 99	18 ± 4*	3.0 ± 0.4*,‡
sPLA$_2$ (ng/dl)	16,553 ± 2,072	242 ± 34*	174 ± 8*
MPO (ng/ml)	365 ± 77	59 ± 9*	62 ± 9*

Values are means ± SEM.
* significantly different from d0 values, at least $P < 0.05$; ‡ significantly different from d21 values, at least $P < 0.05$.

MPO decreases macrophage-to-feces RCT in vivo

Plasma MPO levels increase during the APR, and MPO-mediated oxidative modifications of HDL apolipoproteins, especially apoA-I, have been shown to decrease macrophage cholesterol efflux (28, 29). First, the effect of genetic MPO deficiency on RCT under conditions of acute inflammation was tested. RCT in LPS-injected MPO knockout mice tended to be increased, but did not differ significantly from RCT in LPS-injected controls (supplementary Fig. II). However, murine neutrophils have only 10–20% of the MPO present in the respective human cells (30, 31), so that, especially in the context of a full-blown APR, the relative contribution of MPO might be too small to result in a significant effect. Therefore, we next tested the impact of injecting purified human MPO on RCT. Human MPO administration in the absence of an APR increased plasma levels (527 ± 41 ng/ml, $P < 0.001$), resulted in discrete changes of the plasma lipoprotein profile (**Fig. 5A**), and in the formation of higher molecular mass aggregates of apoA-I upon SDS-PAGE electrophoresis consistent with oxidative modification of the HDL particle (data not shown) (32). Although plasma total cholesterol levels de-

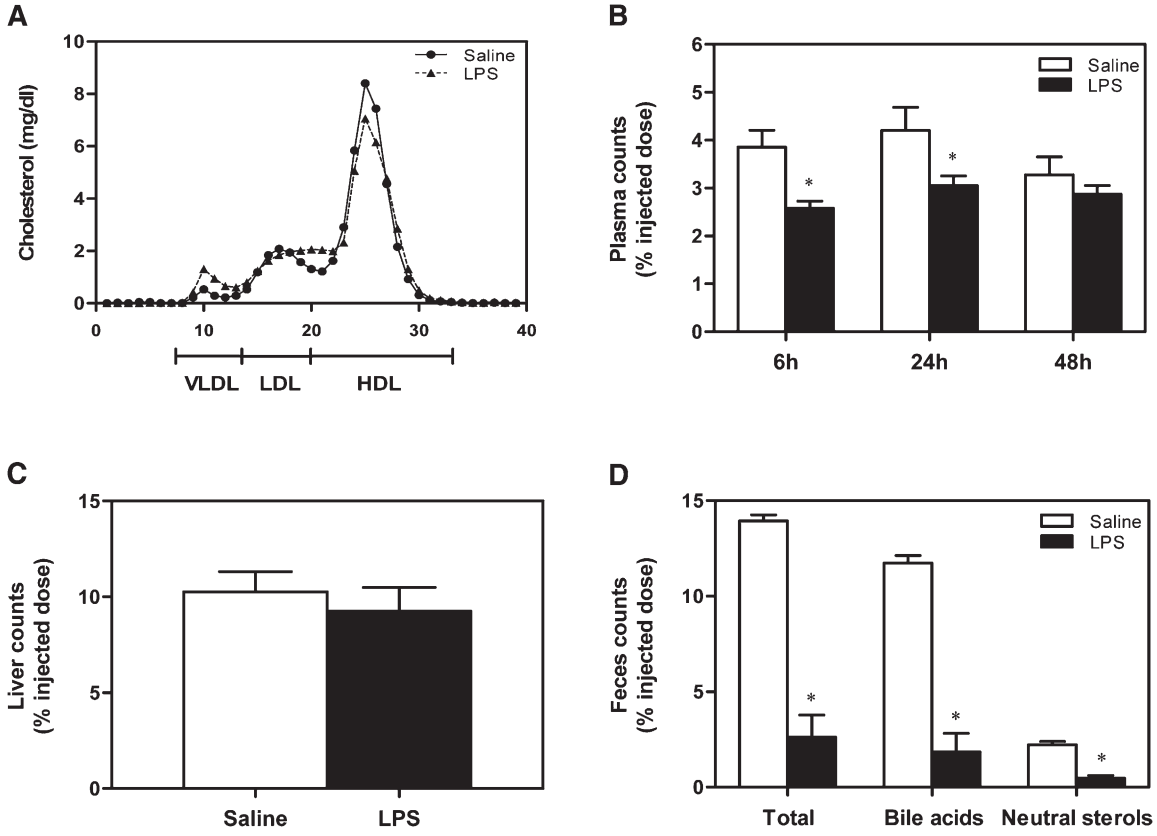

Fig. 2. Acute inflammation impairs macrophage-to-feces reverse cholesterol transport in vivo in mice. C57BL/6J mice received intraperitoneal injections of either 75μg LPS/mouse or saline and [^{3}H]cholesterol-loaded primary mouse macrophage foam cells as detailed in Materials and Methods and were followed for 48 h. A: FPLC cholesterol profiles of pooled plasma samples after injection of either saline (●) or LPS (▲). The relative elution position of the different lipoprotein subclasses is indicated. B: [^{3}H]cholesterol appearance in plasma obtained 6, 24 and 48 h after macrophage administration. C: [^{3}H]cholesterol tracer within liver 48 h after macrophage administration. D: [^{3}H]tracer appearance in total feces collected continuously from 0 to 48 h after macrophage injection and separated into the bile acid and neutral sterol fractions as indicated. Data in B, C, and D are expressed as percentage of the injected tracer dose and are presented as means ± SEM. n = 5–6 for each group. * indicates statistically significant differences from saline controls (at least $P < 0.05$) as assessed by Student's *t*-test.

creased significantly in MPO-injected mice (90 ± 2 vs. 80 ± 2 mg/dl, $P < 0.05$), levels of HDL cholesterol (54 ± 2 vs. 48 ± 5 mg/dl) as well as non-HDL cholesterol (21 ± 1 vs. 20 ± 2 mg/dl) did not differ significantly between groups. Cholesterol tracer recovery in plasma was reduced in MPO-injected mice at 6 h (Fig. 5B) and 24 h ($P < 0.05$). Liver counts were not altered following MPO administration (Fig. 5C). However, total [^{3}H]cholesterol excretion into feces was significantly decreased in the mice injected with MPO compared with controls ($P < 0.05$, Fig. 5D). This was mainly due to a decreased tracer recovery within fecal neutral sterols ($P < 0.05$), whereas tracer within fecal bile acids was not different between the experimental groups. These data show that MPO reduces macrophage-to-feces RCT in vivo, although quantitatively this effect is still substantially lower than the impact of the APR on RCT.

Hepatic cholesterol uptake from acute phase HDL is not altered, but the hepatic gene expression profile in response to LPS indicates decreased biliary secretion

RCT comprises cholesterol efflux toward HDL in the periphery followed by hepatic uptake and excretion into bile. Because the action of three major secreted APPs does not fully explain decreased RCT during the APR, we next assessed whether hepatic cholesterol delivery from acute phase HDL might be impaired. Uptake of [^{3}H]cholesteryl ether from acute phase HDL isolated from sepsis patients (d0) into HepG2 cells was not significantly different compared with HDL obtained after recovery (d21) (8.1 ± 0.7 vs. 7.2 ± 0.5%, n.s.). On the other hand, [^{3}H]cholesteryl ether uptake from control HDL into HepG2 cells treated with a mixture of several pro-inflammatory cytokines was significantly increased (7.7 ± 0.3 vs. 9.8 ± 0.4%, $P < 0.05$). Because these data suggested that the hepatic cholesterol uptake step might not be impaired during inflammation, we next assessed the impact of the APR on the expression levels of key enzymes involved in conversion of cholesterol into bile acids as well as of transporters critical for the biliary secretion process. Hepatic mRNA expression of Cyp27a1, the initial enzyme in the alternative pathway of bile acid synthesis, was decreased by 67% in response to LPS ($P < 0.001$) whereas the expression of two other enzymes involved in bile acid synthesis, namely Cyp7a1, the rate-limiting enzyme for the classic sterol-regulated pathway, and Cyp8b1, the key

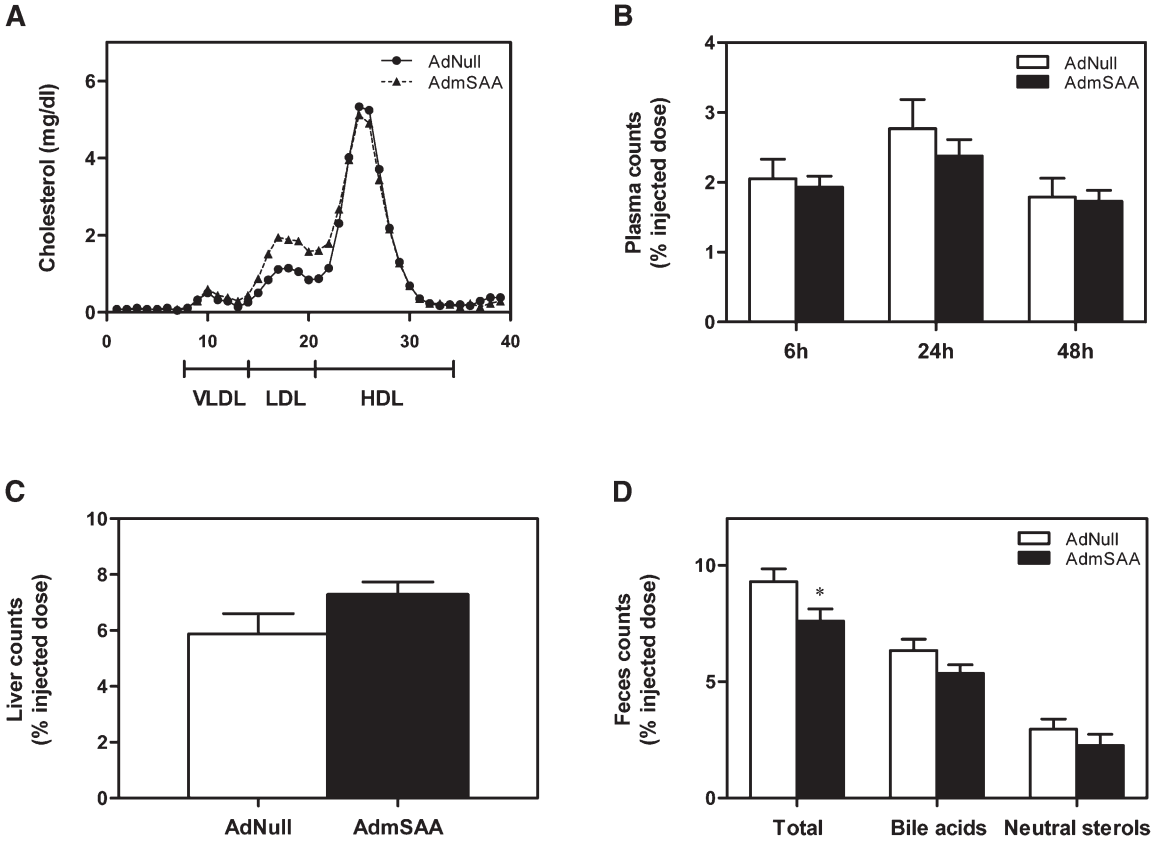

Fig. 3. Mouse SAA overexpression in the absence of systemic inflammation decreases macrophage-to-feces reverse cholesterol transport in vivo in mice. On day 3 after injection with either the control adenovirus AdNull or the mouse CE/J SAA expressing adenovirus AdmSAA C57BL/6J mice received [^{3}H]cholesterol-loaded primary mouse macrophage foam cells as detailed in Materials and Methods and were followed for 48 h. A: FPLC cholesterol profiles of pooled plasma samples after injection of either AdNull (●) or AdmSAA (▲). The relative elution position of the different lipoprotein subclasses is indicated. B: [^{3}H]cholesterol appearance in plasma obtained 6, 24, and 48 h after macrophage administration. C: [^{3}H]cholesterol tracer within liver 48 h after macrophage administration. D: [^{3}H]tracer appearance in total feces collected continuously from 0 to 48 h after macrophage injection and separated into the bile acid and neutral sterol fractions as indicated. Data in C, D, and E are expressed as percentage of the injected tracer dose and are presented as means ± SEM. n = 8 for each group. * indicates statistically significant differences from AdNull injected controls (at least $P < 0.05$) as assessed by Student's *t*-test.

enzyme responsible for synthesis of cholic acid, was decreased, however, not significantly (**Table 2**). Although Sr-b1 expression was not different between LPS-treated mice and saline controls, hepatic mRNA expression of Abcg5 ($P < 0.05$) and Abcg8 ($P < 0.001$), major transport proteins mediating biliary cholesterol secretion, was significantly decreased (Table 2). In addition, expression of Abcb11, an important bile acid export pump, was significantly reduced in response to LPS ($P < 0.001$), whereas the expression of Abcb4, which mediates biliary phospholipid secretion, was unaffected (Table 2). By Western blot (supplementary Fig. III) we confirmed that decreased hepatic mRNA expression during the APR also translates into reduced protein expression of Abcg5 by 65% ($P < 0.05$) and Abcb11 by 36% ($P < 0.05$).

Taken together, these data indicate that coinciding with a substantial reduction in RCT acute inflammation significantly decreases the hepatic expression levels of key enzymes for bile acid synthesis as well as major transport proteins mediating the biliary secretion of bile acids and cholesterol.

DISCUSSION

The results of this study demonstrate that acute inflammation impairs cholesterol efflux to plasma and HDL in patients and macrophage-to-feces RCT in vivo in mice. $sPLA_2$ and MPO but not SAA contribute to reduced cholesterol efflux toward plasma. Although $sPLA_2$ does not represent the underlying basis for the diminished macrophage-to-feces RCT during inflammation, mouse SAA and MPO cause a limited though significant decrease in overall RCT.

Inflammation is closely linked to atherosclerotic CVD on several levels. Chronic inflammatory diseases such as rheumathoid arthritis are typically associated with an increased CVD risk in patients (4, 33), and chronic inflammation causally contributes to atherogenesis in vivo in mouse models (10). On the other hand, plasma levels of several APPs are elevated in patients with atherosclerotic CVD and have been shown to possess a predictive value for future cardiovascular events (8, 9). In addition, acute inflammation impacts on lipid metabolism resulting, in humans, in characteristic changes that resemble a

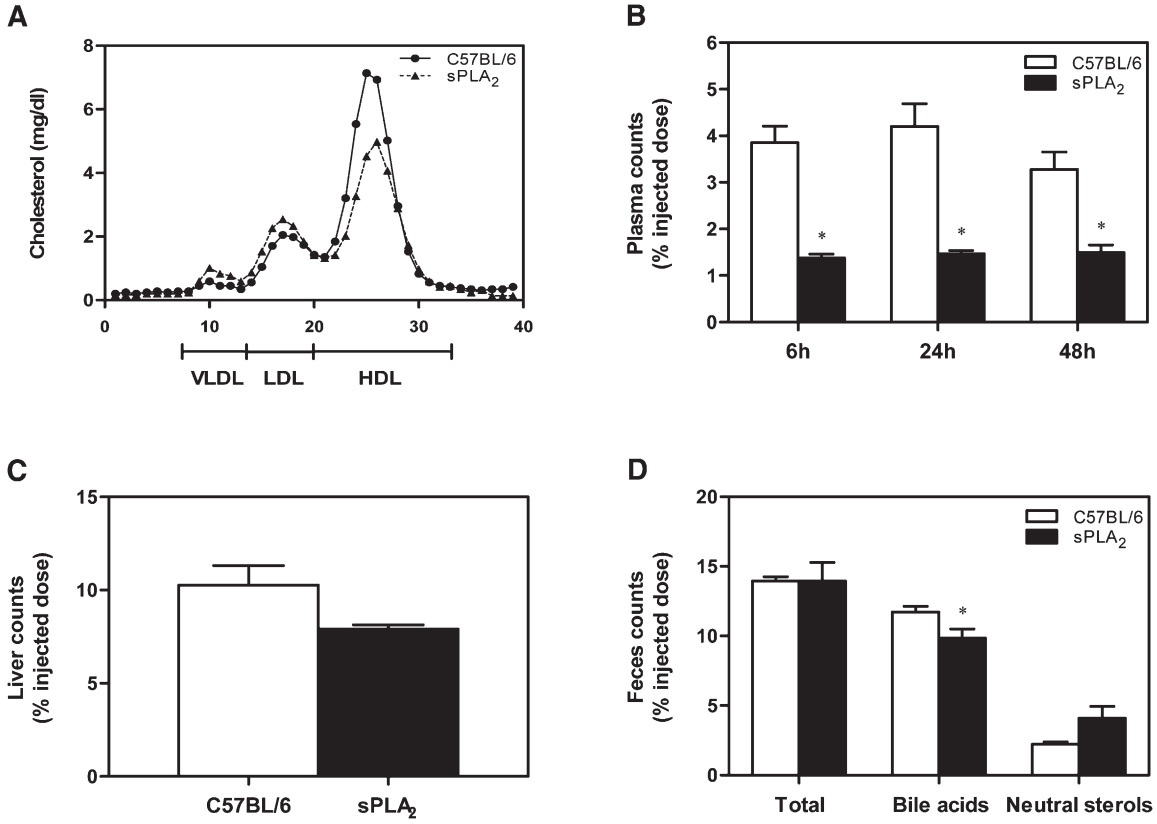

Fig. 4. $sPLA_2$ overexpression in the absence of systemic inflammation does not affect macrophage-to-feces reverse cholesterol transport in vivo in mice. C57BL/6J mice and $sPLA_2$ transgenic mice received [^{3}H]cholesterol-loaded primary mouse macrophage foam cells as detailed in Materials and Methods and were followed for 48 h. A: FPLC cholesterol profiles of pooled plasma samples in C57BL/6J mice (●) and $sPLA_2$ transgenic mice (▲). The relative elution position of the different lipoprotein subclasses is indicated. B: [^{3}H]cholesterol appearance in plasma obtained 6, 24, and 48 h after macrophage administration. C: [^{3}H]cholesterol tracer within liver 48 h after macrophage administration. D: [^{3}H]tracer appearance in total feces collected continuously from 0 to 48 h after macrophage injection and separated into the bile acid and neutral sterol fractions as indicated. Data in B, C, and D are expressed as percentage of the injected tracer dose and are presented as means ± SEM. n = 5–6 for each group. * indicates statistically significant differences from wild-type controls (at least $P < 0.05$) as assessed by Student's *t*-test.

pro-atherogenic lipoprotein profile as it is found in CVD patients, namely reduced plasma HDL cholesterol levels and increased plasma triglycerides (12). In mice, the drop in plasma HDL levels in response to an inflammatory stimulus does not occur as pronounced as in humans and we previously demonstrated using lipoprotein kinetics that the catabolic rates of HDL apolipoproteins as well as HDL cholesteryl ester remain largely unaffected by acute inflammation (11). However, in wild-type mice, inflammation results in altered tissue uptake of HDL and its components, namely significantly increased HDL holoparticle catabolism by the liver and increased selective uptake of HDL cholesteryl ester by the adrenals (11). The adrenals use HDL-derived cholesterol for the production of steroid hormones, which represent an important contributing factor to increased survival during an acute infection (34, 35). Therefore, redistribution of HDL cholesterol during the APR might have proven beneficial during evolution and several APPs might have evolved to aid in directing this process. In addition, the strong decrease in RCT that we observed in response to LPS injection might serve the general purpose of preventing cholesterol loss from the body. While our experiments were in progress, another study was published confirming the basic finding of reduced RCT in response to inflammatory stimuli (35). With regard to the impact of inflammation on cholesterol efflux from macrophages, it also has been shown previously that LPS and pro-inflammatory cytokines downregulate the expression of the two key cholesterol efflux transporters ABCA1 and ABCG1 in macrophages (36, 37). Furthermore, acute phase HDL was demonstrated to not be able to support cholesterol efflux as well as normal HDL (38–40). However, the differential contribution of distinct components of the APR to these results has not been addressed thus far.

In an attempt to delineate the impact of several APPs on RCT in vivo, we first investigated the effect of isolated overexpression of human and mouse SAA in the absence of an APR using recombinant adenoviruses. In plasma, SAA is primarily associated with HDL and can comprise the main apolipoprotein of acute-phase HDL (41, 42). Although earlier reports suggested that SAA might displace apoA-I from the HDL particle (12, 43), thereby potentially accelerating the HDL catabolic rate, overexpression of SAA in vivo by means of a recombinant adenovirus showed no effect on HDL catabolism (24). However, SAA might change

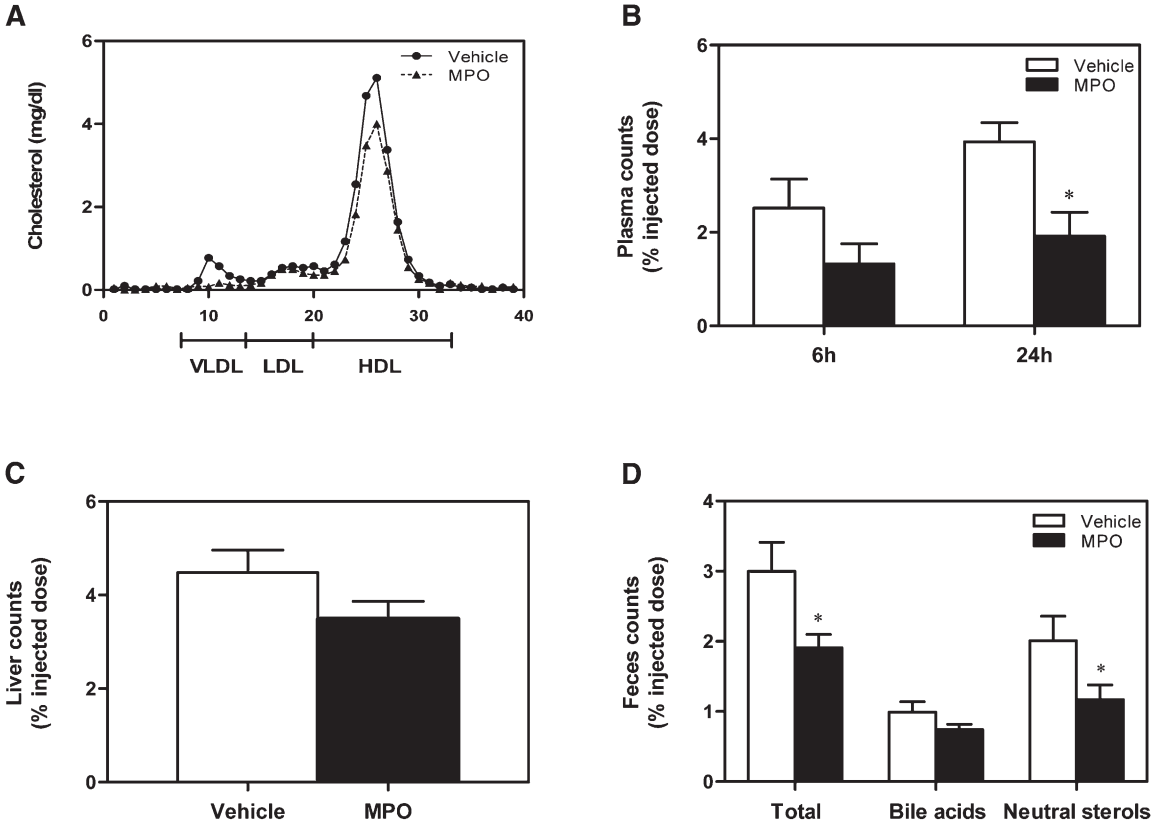

Fig. 5. MPO impairs macrophage-to-feces reverse cholesterol transport in vivo in mice. C57BL/6J mice received [^{3}H]cholesterol-loaded primary mouse macrophage foam cells and consecutive injections in 6 h intervals with either 50 μg/mouse purified MPO or vehicle for a total of 24 h as detailed in Materials and Methods. A: FPLC cholesterol profiles of pooled plasma samples after injection of either vehicle (●) or MPO (▲). The relative elution position of the different lipoprotein subclasses is indicated. B: [^{3}H]cholesterol appearance in plasma obtained 6 and 24 h after macrophage administration. C: [^{3}H]cholesterol tracer within liver 24 h after macrophage administration. D: [^{3}H]tracer appearance in total feces collected continuously from 0 to 24 h after macrophage injection and separated into the bile acid and neutral sterol fractions as indicated. Data in B, C, and D are expressed as percentage of the injected tracer dose and are presented as means ± SEM. n = 6–7 for each group. * indicates statistically significant differences from vehicle controls (at least $P < 0.05$) as assessed by Student's *t*-test.

the functional properties of the HDL particle. Although consistent with the apolipoprotein structure of SAA, this APP is able to mobilize cholesterol from cells (44, 45); SAA in the context of the HDL particle might reduce cholesterol efflux from macrophages (38, 46). Scavenger receptor BI (SR-BI) mediated selective uptake from SAA-enriched HDL is reduced and lipid-free SAA competes with HDL for SR-BI binding, also resulting in decreased selective uptake rates in vitro (47). On the other hand, selective uptake from acute phase HDL containing high levels of SAA was also reported to increase (38). As exemplified in our study, human and mouse SAA might possess different metabolic properties in the context of a murine HDL particle. Although overexpression of human SAA had no impact on tracer appearance in plasma or RCT in vivo, overexpression of mouse SAA decreased overall RCT.

TABLE 2. Hepatic gene expression levels in LPS-injected mice and controls

	Saline (n = 6)	LPS (n = 5)
Cyp27a1	1.00 ± 0.07	0.33 ± 0.10*
Cyp7a1	1.00 ± 0.14	0.67 ± 0.40
Cyp8b1	1.00 ± 0.21	0.53 ± 0.29
Sr-b1	1.00 ± 0.05	0.85 ± 0.15
Abcg5	1.00 ± 0.03	0.62 ± 0.08*
Abcg8	1.00 ± 0.05	0.34 ± 0.05*
Abcb11	1.00 ± 0.06	0.32 ± 0.11*
Abcb4	1.00 ± 0.07	0.76 ± 0.17

mRNA expression levels were determined by real-time quantitative PCR in livers of C57BL/6J mice 48 h after the administration of either saline or LPS as described in Materials and Methods. Results are normalized to the expression of the housekeeping gene cyclophilin and are expressed relative to the respective controls. Data are given as means ± SEM.

* significantly different from saline controls (at least $P < 0.05$).

sPLA$_2$ is an APP with a major effect on HDL catabolism. Transgenic overexpression of human sPLA$_2$ in C57BL/6J mice, a strain lacking the endogenous murine sPLA$_2$ enzyme due to a frameshift mutation, enhances the catabolic rates of HDL cholesteryl ester as well as HDL apoA-I resulting in decreased plasma HDL levels (11, 15, 27). In addition, sPLA$_2$ appears to be a key factor mediating decreased HDL cholesterol levels in response to inflammation. Although LPS injection into wild-type or human apoA-I transgenic mice lacking the endogenous murine sPLA$_2$ enzyme has relatively little effects on HDL cholesterol levels,

LPS administration to the respective models expressing a human $sPLA_2$ transgene that is responsive to inflammatory stimuli results in a pronounced decrease in plasma HDL cholesterol (11, 26). Furthermore, we have also previously established that $sPLA_2$ significantly enhances the redistribution of HDL cholesterol during inflammation, namely causing a strong increase in cholesteryl ester uptake into the adrenals (11), most likely due to increased susceptibility of HDL cholesteryl ester within $sPLA_2$-modified HDL toward SR-BI-mediated selective uptake (21). Although our data indicate that $sPLA_2$ reduces cholesterol efflux toward plasma and might thereby explain the finding of decreased efflux toward plasma from sepsis patients, $sPLA_2$ overexpression does not affect overall in vivo RCT. These results are especially interesting given that transgenic overexpression of human $sPLA_2$ in mice accelerates atherosclerotic lesion development, even on a chow diet (18, 48), and that $sPLA_2$ represents a strong CVD risk factor (49–51).

MPO is an enzyme released from activated neutrophils and monocytes during an inflammatory response and has been implicated in the oxidative modification of HDL apolipoproteins resulting in dysfunctional HDL particles (52, 53). MPO-modified apoA-I and HDL have a reduced ability to elicit cholesterol efflux from macrophages (28, 29), consistent with the decrease in tracer appearance in plasma in response to MPO injection observed in our current study. In addition, MPO decreases the ability of apoA-I to activate LCAT (54). On the other hand, hypochlorite modification of HDL mimicking the enzymatic activity of MPO has been shown to also decrease SR-BI-mediated selective uptake from oxidized HDL (32). These MPO-induced changes have been suggested to translate into decreased RCT; however, to our knowledge, the present study is the first to demonstrate significantly reduced RCT in response to MPO using an integrated physiologically relevant in vivo RCT assay. In humans, reduced RCT might therefore conceivably represent a contributing factor to the tight association between plasma MPO levels and risk of atherosclerotic CVD in several population studies (53–55). However, in mice the absence of MPO did not affect RCT in response to an APR, pointing toward a different mechanistic basis for decreased RCT during LPS-induced inflammation in the murine model.

Of note, mice do not express the cholesteryl ester transfer protein that plays an important role in human lipoprotein metabolism and also in RCT. Cholesteryl ester transfer protein expression is downregulated by inflammation (56, 57), which conceivably might represent an additional factor impacting on RCT in humans that was not addressed in the present study.

As a general scheme, our results suggest that plasma APPs contribute to a certain extent to decreased RCT during an APR but do not fully explain it. Rather, APPs appear to be responsible for preserving the cholesterol content of peripheral tissues by reducing efflux and for mediating the redistribution of tissue cholesterol uptake from lipoproteins, especially HDL. The liver apparently serves in the setting of the APR as a gatekeeper to prevent loss of sterols from the body. This is, on the one hand, achieved by downregulation of the enzymes required for the conversion of cholesterol to bile acids (58, 59) and on the other hand, by decreasing the expression of Abcg5/g8 and Abcb11, responsible for the largest part of biliary secretion of cholesterol and bile acids, respectively. The decreased expression of Abcg5 and Abcb11 on the mRNA and protein level observed in our study is consistent with previously published findings (35, 37) and translates also into an actual reduction in biliary secretion rates of cholesterol and bile acids during the APR in mice (unpublished observations).

In summary, our data demonstrate that reduced cholesterol efflux observed in patients with acute inflammation could at least partially be attributed to increased plasma levels of $sPLA_2$ and MPO. Although in mice, $sPLA_2$ did not influence overall RCT, murine SAA and MPO injection resulted in a limited decrease in RCT from macrophages to feces in vivo. However, also reduced biliary secretion of bile acids and cholesterol by the liver appears to be a major contributing factor to decreased macrophage-to-feces RCT during the APR.

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