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Lack of LCAT reduces the LPS-neutralizing capacity of HDL and enhances LPS-induced inflammation in mice

Peristera-Ioanna Petropoulou [a,1], Jimmy F.P. Berbée [a,1], Vassilios Theodoropoulos [a], Aikaterini Hatziri [a], Panagiota Stamou [b], Eleni A. Karavia [a], Alexandros Spyridonidis [b], Iordanes Karagiannides [a,c], Kyriakos E. Kypreos [a,*]

[a] Pharmacology Department, University of Patras Medical School, Rio Achaias TK. 26500, Greece
[b] Hematology Department, University of Patras Medical School, Rio Achaias, TK. 26500, Greece
[c] Inflammatory Bowel Disease Center, Neuroendocrine Assay Core, Division of Digestive Diseases, David Geffen School of Medicine at UCLA, Los Angeles, California, USA

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ABSTRACT

HDL has important immunomodulatory properties, including the attenuation of lipopolysaccharide (LPS)-induced inflammatory response. As lecithin–cholesterol acyltransferase (LCAT) is a critical enzyme in the maturation of HDL we investigated whether LCAT-deficient (*Lcat*$^{-/-}$) mice present an increased LPS-induced inflammatory response. LPS (100 μg/kg body weight)-induced cytokine response in *Lcat*$^{-/-}$ mice was markedly enhanced and prolonged compared to wild-type mice. Importantly, reintroducing LCAT expression using adenovirus-mediated gene transfer reverted their phenotype to that of wild-type mice. *Ex vivo* stimulation of whole blood with LPS (1–100 ng/mL) showed a similar enhanced pro-inflammatory phenotype. Further characterization in RAW 264.7 macrophages *in vitro* showed that serum and HDL, but not chylomicrons, VLDL or the lipid-free protein fraction of *Lcat*$^{-/-}$ mice, had a reduced capacity to attenuate the LPS-induced TNFα response. Analysis of apolipoprotein composition revealed that LCAT-deficient HDL lacks significant amounts of ApoA-I and ApoA-II and is primarily composed of ApoE, while HDL from *Apoa1*$^{-/-}$ mice is highly enriched in ApoE and ApoA-II. ApoA-I-deficiency did not affect the capacity of HDL to neutralize LPS, though *Apoa1*$^{-/-}$ mice showed a pronounced LPS-induced cytokine response. Additional immunophenotyping showed that *Lcat*$^{-/-}$, but not *Apoa1*$^{-/-}$ mice, have markedly increased circulating monocyte numbers as a result of increased Cd11b^{+}Ly6C^{med} monocytes, whereas 'pro-inflammatory' Cd11b^{+}Ly6C^{hi} monocytes were reduced. In line with this observation, peritoneal macrophages of *Lcat*$^{-/-}$ mice showed a markedly dampened LPS-induced TNFα response. We conclude that LCAT-deficiency increases LPS-induced inflammation in mice due to reduced LPS-neutralizing capacity of immature discoidal HDL and increased monocyte number.

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1. Introduction

High-density lipoprotein (HDL) is a heterogeneous mixture of lipoprotein particles that differ in size, shape, lipid and apolipoprotein composition. HDL is continuously remodeled by lipolytic enzymes and lipid transporters but also by exchanging lipids and apolipoproteins with other lipoproteins and tissues [1,2]. Mature HDL particles consist of a hydrophobic core containing cholesteryl esters and triglycerides and a surface lipid monolayer composed mainly of phospholipids and free cholesterol, where amphipathic apolipoproteins are embedded. Apolipoprotein (Apo) A-I and ApoA-II are the two main protein components of HDL, but also other apolipoproteins and enzymes such as apolipoprotein E (ApoE) and lecithin–cholesterol acyltransferase (LCAT), are associated with HDL [1].

HDL possesses several important biological functions [3]. Its best characterized function is its role in 'reverse cholesterol transport'. HDL takes up cholesterol from the peripheral tissues, including the vessel wall, to deliver it to the liver for excretion into the bile. In addition, HDL has the ability to alleviate inflammation and suppress immune activation, which is critical for the prevention of atherosclerosis and coronary heart disease [4]. Indeed, the anti-inflammatory properties of HDL contribute significantly to its atheroprotective potential as persistent low-grade inflammation is a key factor in the development and progression of atherosclerosis.

Abbreviations: ApoA-I, apolipoprotein A-I; ApoA-II, apolipoprotein A-II; ApoE, apolipoprotein E; AdGFP, adenovirus expressing the green fluorescence protein; AdLCAT, adenovirus expressing the LCAT protein; GM-CSF, granulocyte–macrophage colony stimulating factor; HDL, high-density lipoprotein; IL-2, interleukin 2; IL-12p70, interleukin 12 subunit p70; IFN-γ, interferon gamma; IL-10, interleukin 10; LCAT, lecithin–cholesterol acyltransferase; LPS, lipopolysaccharide; TNFα, tumor necrosis factor alpha; TRL, triglyceride-rich lipoproteins; WBC, white blood cells; WT, wild-type.

* Corresponding author at: University of Patras Medical School, Rio, Achaia, PC. 26500, Greece.
E-mail address: kkypreos@med.upatras.gr (K.E. Kypreos).

[1] Equal contribution to this work.

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Low circulating levels of the bacterial endotoxin, lipopolysaccharide (LPS) appear to sustain a non-resolving mild inflammation. As a consequence, low-grade endotoxemia may skew the immune milieu of the host towards a low-grade pro-inflammatory state, which ultimately leads to sepsis [5], a major cause of death in intensive care patients. LPS, the major pathogenic mediator of Gram-negative sepsis, is mainly responsible for the observed mortality through its cytotoxic functions [6]. LPS is recognized by the Toll-like receptor 4 (TLR4) on the cell surface of mainly monocytes and macrophages [7], and after binding a signaling cascade initiates the secretion of an array of cytokines, including TNFα. To prevent damage to tissues and organs by the LPS-mediated inflammatory response, the host has developed several control mechanisms that include inhibitory LPS-binding proteins [8] and plasma lipoproteins, such as HDL [9].

In humans with severe sepsis plasma HDL-C levels decrease rapidly [10]. Concomitantly, structural alteration of HDL, including a significant increase in serum amyloid-A (SAA) levels, takes place at the beginning of sepsis. During recovery, plasma HDL-C levels are restored and SAA is slowly replaced by ApoA-I and other apolipoproteins [10]. Low HDL-C levels inversely correlate with the severity of septic disease and associate with a magnified systemic inflammatory response [11], although it is difficult to distinguish whether changes in plasma lipoproteins simply mirror the severity of disease or they directly alter the host response to inflammation. In healthy individuals, low HDL-C levels are associated with heightened inflammatory response on endotoxin challenge compared to subjects with normal or high HDL-C levels [12], without differences in the HDL proteome [13]. In agreement with clinical data, *Apoa1*$^{-/-}$ mice, which lack classical HDL, exhibit a lower LPS-neutralizing capacity in serum compared with serum from control mice [14], whereas transgenic overexpression of human ApoA-I moderately improves survival confirming that HDL elevation may protect against septic death [15].

Several mechanisms have been described to contribute to the HDL-mediated protection. It has been proposed that plasma lipoproteins including HDL bind and neutralize Gram-positive bacterial lipoteichoic acid (LTA) [16] as well as Gram-negative bacterial LPS [17]. In addition, HDL may protect against sepsis by enabling LPS clearance *via* its interaction with Scavenger Receptor BI (SR-BI) [18]. In fact, almost all LPS is present as an LPS–HDL complex in blood [19,20]. A third mechanism includes the release of macrophage-bound LPS by HDL that in turn reduces macrophage activation [21]. Another potential mechanism is the facilitation of an early inflammatory response to Gram-negative bacteria *via* suppression of the inhibitory activity of LBP [22].

LCAT is a critical enzyme in the maturation of HDL by mediating the conversion of discoidal to spherical HDL [23]. However, the role of LCAT or LCAT-mediated maturation of HDL in the neutralizing capacity of HDL is unknown. Therefore, in the present study we investigated whether lack of mature HDL in *Lcat*$^{-/-}$ mice increases the LPS-induced inflammatory response, and compared the response to that of *Apoa1*$^{-/-}$ mice. We show that *Lcat*$^{-/-}$ mice on one hand have an increased overall systemic pro-inflammatory response as a result of decreased LPS-neutralizing capacity, but on the other hand have a reduced pro-inflammatory monocyte/macrophage phenotype that partly opposes its pro-inflammatory effect. Marked differences were observed between *Apoa1*$^{-/-}$ and *Lcat*$^{-/-}$ mice suggesting that qualitative alterations in HDL may influence inflammatory response through distinct mechanisms.

2. Materials and methods

2.1. Animals

Lcat$^{-/-}$ mice (kind donation from Prof. Silvia Santamarina-Fojo) and *Apoa1*$^{-/-}$ and C57BL/6 mice (both from Jackson Laboratory, Bar Harbor, Maine, USA) were bred in the animal facility of the University of Patras. Male mice of 10–16 weeks of age were used for experiments. Mice were allowed unrestricted access to food and water under a 12 h light/dark cycle. At the end of each experiment, mice were sacrificed and plasma and tissue samples were collected, snap-frozen in liquid nitrogen, and stored at −80 °C. All animal studies were governed by the EU guidelines of the *Protocol for the Protection and Welfare of Animals* and conducted in accordance with the Declaration of Helsinki and authorized by the appropriate committee of the Laboratory Animal Center of the University of Patras Medical School and the Veterinary Authority of the Prefecture of Western Greece.

2.2. In vivo LPS stimulation

Mice were injected intravenously with *Escherichia coli* LPS 055:B5 (Sigma, St. Louis, MO, USA; 100 μg/kg body weight) and PBS as a vehicle. Before the injection (t = 0) and at the indicated time points, blood samples were taken from the tail vein into EDTA-capillary tubes and stored on ice. Plasma was isolated and plasma cytokine levels were determined using the Bio-Plex Mouse Cytokine Group I 13-plex Assay, with Magnetic Beads (Bio-Rad, Hercules, CA, USA). Final data were obtained and analyzed *via* the Bio-plex 3D Suspension array system (Bio-Rad). Absolute quantitation was achieved *via* cytokine specific standards for curve creation provided with the kit. TNFα was also measured with ELISA using the commercially available mouse TNF alpha ELISA kit (eBioscience, San Diego, USA).

2.3. In vivo AdLCAT administration

The amplification and expansion of the attenuated recombinant control adenovirus AdGFP and the LCAT-expressing adenovirus AdLCAT, have been described previously [24]. Typically, titers of approximately 2×10^{10} pfu/mL were obtained for each virus preparation. *Lcat*$^{-/-}$ mice were injected with either AdGFP or AdLCAT adenovirus at a low dose of 5×10^{8} pfu. As an additional control, WT mice were injected with AdGFP. Three days post-infection, samples of blood were collected for baseline plasma cholesterol and TNFα levels. Immediately after, all mice were injected intravenously with LPS (100 μg/kg body weight). TNFα response was monitored in plasma at the indicated time points as described above.

2.4. Western blot analysis

Western blot analysis of ApoA-I, ApoE and ApoA-II in HDL fraction was performed as described previously [24]. As primary antibodies a goat anti-human ApoA-I antibody (Biodesign International, Saco, ME, USA), a goat anti-human ApoE antibody (Biodesign International), and a goat-anti-human ApoA-II antibody (Biodesign International) were used, and as secondary antibody a rabbit anti-goat antibody (Santa-Cruz, Dallas, TX, USA).

2.5. Ex vivo whole blood LPS stimulation

Whole blood was collected into EDTA-capillary tubes, diluted 1:25 with serum-free DMEM medium and plated onto 24-well plates. The blood was incubated for 18 h with or without LPS (1 and 10 ng/mL) and subsequently transferred into a tube and centrifuged at 1000 rpm for 5 min for removal of the blood cells. TNFα levels were quantified by ELISA as described above.

2.6. Fractionation of serum lipoproteins by density gradient ultra centrifugation

For the isolation of serum, blood from unfasted mice was collected into Eppendorf tubes on ice. Subsequently, blood samples were incubated for 15 min at 37 °C to activate clotting and centrifuged for 10 min at 7000 rpm. Pooled serum per group was fractionated by KBr density

gradient ultracentrifugation. The triglyceride-rich lipoproteins (TRL; chylomicrons and VLDL) were isolated from the top fraction with a density of 1.006 g/L, the HDL from the 1.063 g/L fraction, and the lipoprotein-free proteins from the bottom fraction with a density of 1.21 g/L.

2.7. Lipid and total protein quantitation in plasma, serum and lipoprotein fractions

Total cholesterol (TC; Cholesterol FS kit; DiaSys Diagnostic Systems GmbH, Holzheim, Germany), free cholesterol (FC; Free Cholesterol E Kit; Wako, Richmond, VA, USA) and phospholipids (PL; Phospholipid C determination kit; Wako) were determined spectrophotometrically according to manufacturers' instructions. Total protein in lipoprotein fractions or cell lysates was measured by the Lowry assay using the DC™ Protein Assay Kit (Bio-Rad). The lipid content of the HDL fraction are expressed as mg/dL of the original serum sample.

2.8. FACS analysis for phenotyping of monocytes

Blood from WT, *Lcat*$^{-/-}$ and *Apoa1*$^{-/-}$ mice was collected into EDTA-capillary tubes. 25 μL were transferred in a new tube and incubated on ice with 0.5 μg of FCR III/II blocking antibody (anti-mouse CD16/32; eBioscience) for 15 min. Cells were washed and incubated with 0.25 μg of anti-mouse CD11b-FITC (eBioscience), 0.125 μg of anti-mouse Ly6C APC (eBioscience), for 20 min. Afterwards, erythrocytes were lysed with an ammonium chloride-containing lysis buffer. At least twenty thousand events were acquired using a BD FACSCalibur™ flow cytometer (Becton-Dickinson, USA) and data were analyzed using Flowjo software (Tree Star). All living cells were gated from the FSC/SSC scatter plot and indicated as total white blood cells (WBC). From these total WBC we plotted CD11b^{+} *versus* Ly6C cells and determined the percentage of Ly6C^{lo}, Ly6C^{med,} and Ly6C^{hi} within CD11b^{+} monocytes and expressed them as percentage of total WBC.

2.9. In vitro macrophage LPS stimulation

RAW 264.7 cells, a murine macrophage cell line, were seeded onto 96-well plates (60×10^3 cells/well) and cultured overnight at 37 °C in DMEM containing 10% FBS and 1% pen/strep. For the isolation of primary peritoneal macrophages, mice were initially injected intraperitoneally with 1 mL of 3% (w/v) Brewer's thioglycollate medium. Four days later, peritoneal macrophages were obtained by peritoneal lavage with 10 mL ice-cold sterile PBS using a 25G needle. Following 2 cycles of washing with ice-cold sterile PBS, cells were resuspended into 1 mL DMEM culture medium containing 10% fetal bovine serum (FBS) and 1% pen/strep and plated onto 24 well plates (8×10^5 cells/well). The next day, macrophages were washed with serum-free DMEM medium and incubated with LPS (1–100 ng/mL) that was preincubated (30 min 37 °C) with or without serum or lipoprotein fractions from WT, *Lcat*$^{-/-}$ and *Apoa1*$^{-/-}$ mice in DMEM supplemented with 0.1% human serum albumin (4 h at 37 °C) [25,26]. As controls, cells were incubated with human serum albumin as vehicle and serum or lipoprotein fractions at the highest concentration used. The medium was collected and TNFα was determined in the cultured medium by ELISA as described above. For the experiments involving peritoneal macrophages protein content of each cell lysate was determined as described above and the TNFα levels were expressed as ng/mg cell protein.

2.10. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from fresh frozen liver tissue using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Reverse transcription was performed using the PrimeScriptTM RT reagent kit (Takara Bio Inc., Otsu, Shiga, Japan). Real-Time PCR for *F4/80*, *Cd68*, *Mcp1*, *Mrc1*, *Cd163*, *Arg1* and the housekeeping genes *Rpl13A*, *Rplp0* was performed in a MicroAmp® 96-well reaction plate (Applied Biosystems, Foster City, CA, USA) using the KAPA SYBR® FAST Universal qPCR kit (Kapa Biosystems, Woburn, MA, USA), in an Applied Biosystems® StepOne™ cycler (Applied Biosystems). Primers (Invitrogen-Life Technologies, Renfrewshire, Scotland) used are shown in Table 1. Data were normalized for *Rpl13A*, *Rplp0* expression.

2.11. Statistical analysis

Data are reported as mean ± SEM. * and # indicate $p < 0.05$, ** and ## indicate $p < 0.01$, and ### and *** indicate $p < 0.001$. Non-significant differences are not indicated. *n* indicates the number of animals tested in each experiment. Statistical analyses between 2 groups were assessed using unpaired two-tailed Student's *t*-test and between 3 groups using one-way ANOVA with a Bonferroni's post-hoc test. GraphPad Prism 5 was used for these statistical analyses.

3. Results

3.1. Baseline HDL-lipid and apolipoprotein characterization of HDL

Baseline serum lipid and HDL-lipid levels of WT, *Lcat*$^{-/-}$ and *Apoa1*$^{-/-}$ mice are shown in Table 2. As expected *Lcat*$^{-/-}$ mice carry only minimal amounts of cholesteryl esters (CE) in their HDL and the predicted relative size of HDL is also much smaller as compared to HDL of WT mice. The HDL-lipid composition of *Apoa1*$^{-/-}$ mice shows less pronounced differences with the HDL of WT mice. To examine the qualitative differences in the protein composition of HDL among the 3 genotypes we performed western blot analysis for the three most abundant HDL apolipoproteins, *i.e.* ApoA-I, ApoE and ApoA-II. HDL of WT mice consists almost exclusively of ApoA-I, while LCAT-deficient HDL contains barely detectable ApoA-I, but primarily ApoE (Fig. 1). ApoA-I-deficient HDL consists mainly of ApoE and to a lesser extent of ApoA-II.

3.2. LCAT-deficiency enhances the LPS-induced inflammatory response in vivo

To assess the potential effects of these qualitative alterations in HDL on its anti-inflammatory properties, we first examined whether LCAT-deficiency modulates the systemic inflammatory response *in vivo*

Table 1
Sequence of oligonucleotide primers used in the qRT-PCR analysis.

Mouse gene	Marker for	Forward sequence	Reverse sequence
F4/80	Common macrophage	5′- CTTTGGCTATGGGCTTCCAGTC	5′- GCAAGGAGGACAGAGTTTATCGTG - 3′
Cd68	Common macrophage	5′- ATCCCCACCTGTCTCTCTCA - 3′	5′- TTGCATTTCCACAGCAGAAG - 3′
Mcp1	Common-monocyte attraction	5′- GCATCTGCCCTAAGGTCTTCA - 3′	5′- TTCACTGTCACACTGGTCACTCCTA - 3′
Mrc1	M2	5′- GAGAGCCAAGCCATGAGAAC - 3′	5′- GTCTGCACCCTCCGGTACTA - 3′
Cd163	M2	5′- CTCAGGAAACCAATCCCAGA - 3′	5′- CAAGAGCCCTCGTGGTAGAC - 3′
Arg1	M2	5′- GGTTCTGGGAGGCCTATCTTACA - 3′	5′- TCTTCACCTCCTCTGCTGTCTTC - 3′
Rplp0	Housekeeping gene	5′- GGACCCGAGAAGACCTCCTT - 3′	5′- GCACATCACTCAGAATTTCAATGG - 3′
Rpl13a	Housekeeping gene	5′- CTGGTACTTCCACCCGACCTC - 3′	5′- GGATCCCTCCACCCTATGACA - 3′

Table 2
Serum and HDL lipid levels.

	WT	*Lcat*$^{-/-}$	*Apoa1*$^{-/-}$
Serum lipid levels			
TC (mg/dL)	64.85 ± 3.27	30.21 ± 1.19	38.40 ± 0.90
PL (mg/dL)	166.23 ± 1.78	82.50 ± 1.41	91.76 ± 1.30
TG (mg/dL)	58.68 ± 2.80	101.80 ± 3.08	37.47 ± 1.80
Serum HDL-lipid levels			
HDL–TC (mg/dL)	38.02 ± 0.04	3.38 ± 0.04	18.26 ± 0.05
HDL–FC (mg/dL)	10.33 ± 0.05	2.70 ± 0.18	5.39 ± 0.13
HDL–CE (mg/dL)	27.69 ± 0.02	0.68 ± 0.46	12.87 ± 0.04
HDL–PL (mg/dL)	16.98 ± 0.08	4.80 ± 0.17	14.00 ± 0.10
HDL–TG (mg/dL)	1.78 ± 0.61	2.23 ± 0.30	1.25 ± 0.19
HDL–Protein (mg/dL)	195 ± 3	47 ± 4	112 ± 3
Relative HDL size (TG + CE)/(PL + TC)	1.08	0.39	0.73

Serum lipid levels are presented as mean ± SEM, n = 5 mice per genotype. Serum HDL-lipid levels are presented as mean ± SEM, n = 3 technical replicates of isolated HDL from pooled serum of 5 mice per genotype. CE, cholesteryl esters; FC, free cholesterol; PL, phospholipids; TC, total cholesterol; TG, triglycerides.

upon stimulation with a low dose of LPS (*i.e.* 100 μg/kg body weight). Based on cytokine profile, *Lcat*$^{-/-}$ mice showed a clear pro-inflammatory response when compared to their WT counterparts. The pro-inflammatory cytokines TNFα, IFNγ, IL-2, IL-12p70, the growth factor GM-CSF and the anti-inflammatory cytokine IL-10 were all significantly elevated after LPS injection (Fig. 2 A–F). Interestingly, *Lcat*$^{-/-}$ mice also display a prolonged response that fails to return to baseline levels even 240 min after initial challenge with LPS. This is a strikingly different response from WT mice that show a transient and less pronounced increase in their LPS-induced cytokine response, with several cytokines returning to baseline levels within the timeframe of the study. Interestingly, the enhanced LPS-induced TNFα response of *Lcat*$^{-/-}$ mice appears comparable to that of *Apoa1*$^{-/-}$ mice that are known to develop a poor response to LPS (Fig. 2 G–H).

We next validated whether adenoviral expression of human LCAT could restore the LPS-induced pro-inflammatory phenotype in *Lcat*$^{-/-}$ mice. We first confirmed that transfection of *Lcat*$^{-/-}$ mice with AdLCAT indeed normalized the plasma lipoprotein profile to a similar extent as observed in WT mice (Table 3). Importantly, reintroducing LCAT in *Lcat*$^{-/-}$ mice normalized the LPS-induced TNFα response to a similar extent as observed in WT mice (Fig. 2 I).

Since LCAT is a plasma enzyme that primarily modifies circulating lipoproteins, we next investigated if the apparent changes in the inflammatory profile of *Lcat*$^{-/-}$ mice are due to changes in blood. Indeed, the enhanced pro-inflammatory response was retained in whole blood of *Lcat*$^{-/-}$ mice since *ex vivo* stimulation of whole blood of *Lcat*$^{-/-}$ mice with LPS resulted in a markedly increased LPS-induced TNFα response that was even greater than the response observed in whole blood isolated from *Apoa1*$^{-/-}$ mice (Fig. 3).

3.3. LCAT-deficient serum and HDL enhance the LPS-induced pro-inflammatory response in vitro

The LPS-induced inflammatory response by whole blood may be determined by both serum components able to modulate inflammatory response, such as lipoproteins, as well as by the number and activity of monocytes, the effector cells. To characterize the effects of serum and its components on LPS inflammation, RAW 264.7 macrophages were stimulated with LPS in the presence of serum from *Lcat*$^{-/-}$ mice as well as serum from WT and *Apoa1*$^{-/-}$ mice, as controls. LCAT-deficient serum clearly stimulated the LPS-induced TNFα response at low concentrations of serum (≤0.1%) and only at high concentrations (10%) was able to neutralize this response (Fig. 4 A). This impaired LPS-neutralizing capacity of LCAT-deficient serum was comparable to the one observed with ApoA1-deficient serum (Fig. 4 B). As expected serum from WT mice caused a dose-dependent reduction in LPS-induced TNFα response that was apparent even at very low concentrations.

To further dissect which serum components are responsible for the observed effect, we fractionated plasma by KBr density gradient ultracentrifugation and investigated the LPS-neutralizing capacity of various serum lipoproteins and the lipid-free protein fraction. As expected, HDL from WT mice neutralized LPS-induced inflammation. This neutralizing effect was abolished when HDL from LCAT-deficient mice was used (Fig. 4 C). These data may suggest that *Lcat*$^{-/-}$ mice not only lack mature HDL in their serum but also that the LPS-neutralizing capacity still present in the remaining discoidal HDL may be impaired. Further analysis indicated that the increased LPS-induced inflammatory response of the LCAT-deficient serum could not be explained by an impaired response of triglyceride-rich lipoproteins (TRL; chylomicrons and VLDL) and the lipid-free serum proteins (Fig. 4 C). Surprisingly, the TRL fraction of *Lcat*$^{-/-}$ serum was rather anti-inflammatory suggesting that the pro-inflammatory properties of *Lcat*$^{-/-}$ plasma are solely due to HDL. Similar but less pronounced results were obtained when lipoproteins from *Apoa1*$^{-/-}$ serum were tested, with the exception of TRLs which failed to neutralize LPS (Fig. 4 D).

A possible explanation is that LCAT serves as a bridging molecule that binds LPS onto HDL, thus Lcat$^{-/-}$ HDL fails to neutralize LPS in circulation due to lack of LCAT. To test this possibility, we incubated LCAT-deficient HDL with purified LCAT protein and measured its anti-inflammatory potential. Our data show that incubation of LCAT with *Lcat*$^{-/-}$ HDL did not affect LPS-induced pro-inflammatory response (Fig. 4 E), excluding a direct effect of LCAT protein as a bridging molecule, on LPS-induced inflammation.

3.4. LCAT-deficiency associates with high monocyte numbers, but reduced inflammatory status of monocytes and macrophages

To characterize the possible role of circulating monocytes and tissue macrophages to the observed pro-inflammatory phenotype *in vivo*, we investigated circulating levels of CD11b-positive (CD11b^{+}) monocytes and their inflammatory status, *i.e.* Ly6C expression. We found increased numbers of circulating CD11b^{+}/Ly6C^{+} monocytes (Fig. 5 A) in *Lcat*$^{-/-}$ mice that were unexpectedly due to an increase in normal circulating Ly6C^{med} monocytes, and not in newly recruited Ly6C^{hi} monocytes (Fig. 5 B,D–F). Increased number of Ly6C^{hi} monocytes is a usual marker of a heightened systemic inflammatory condition of the mouse [27]. Indeed, the Ly6C^{hi}/Ly6^{med} ratio was decreased by 2.5-fold in *Lcat*$^{-/-}$ mice (Fig. 5 C), compared to WT counterparts. Interestingly, in line with this

Table 3
Total and esterified plasma cholesterol of WT and *Lcat*$^{-/-}$ mice treated with AdGFP and *Lcat*$^{-/-}$ mice treated with AdLCAT.

Group	TC day 0 (mg/dL)	TC day 3 (mg/dL)	% change	P (day 0 *vs* day 3)	CE day 0 (mg/dL)	CE day 3 (mg/dL)	% change	P (day 0 *vs* day 3)
(A) WT + AdGFP	59.4 ± 5.2	58.3 ± 6.2	−2%	0.90	43.4 ± 4.6	39.9 ± 4.4	−9%	0.60
(B) *Lcat*$^{-/-}$ + AdGFP	23.4 ± 4.1	17.7 ± 2.4	−32%	0.26	11.1 ± 3.2	7 ± 2	−59%	0.31
(C) *Lcat*$^{-/-}$ + AdLCAT	25.7 ± 2.9	51.2 ± 5.8	+50%	0.01	17 ± 3.2	39.6 ± 5.3	+57%	0.01
P (A *vs* B)	<0.001	<0.001			<0.001	<0.001		
P (A *vs* C)	<0.01	0.43			<0.01	0.96		
P (B *vs* C)	0.68	<0.01			0.24	<0.001		

% change refers to changes in plasma lipid levels prior and 3 days following infection. Data are presented as mean ± SEM, n = 5–6 mice per group. CE, cholesteryl ester; TC, total cholesterol.

Table 4
Summary of lipid- and inflammation-related features of WT, *Lcat*$^{-/-}$ and *Apoa1*$^{-/-}$ mice.

	WT	*Lcat*$^{-/-}$	*Apoa1*$^{-/-}$
General features			
HDL	Mature	Immature	Mature/atypical
ApoA-I on HDL	√	×	×
ApoE on HDL	×	√	√
ApoA-II on HDL	×	×	√
In vivo cytokine response to LPS	Normal	↑	↑
Pro-inflammatory features			
Ex vivo cytokine response to LPS of whole blood	Normal	↑↑	↑
In vitro LPS-neutralizing potential of serum	Normal	↓↓	↓
In vitro LPS-neutralizing potential of HDL	Normal	↓↓	Normal
Blood monocyte	Normal	↑↑	Normal
Anti-inflammatory features			
Blood Ly6C$^{hi/med}$ monocyte ratio	Normal	↓↓	Normal
CD11b expression	Normal	↓↓	Normal
In vitro macrophage response to LPS	Normal	↓↓	↑

↑↑ indicates large increase compared to WT group; ↑ indicates moderate increase compared to WT group; ↓↓ indicates large decrease compared to WT group; ↓ indicates moderate decrease compared to WT group.

reduced inflammatory status of circulating monocytes, we also observed a consistent reduction in CD11b expression (evident by reduced Mean Fluorescence Intensity) in all three monocyte populations of *Lcat*$^{-/-}$ mice (Fig. 5 G–J). To our surprise, circulating monocytes of *Apoa1*$^{-/-}$ mice showed a distinctly different pattern. The total monocyte number was slightly increased compared to WT mice (Fig. 5 A), while no differences were observed in the number of Ly6C monocyte subsets (Fig. 5 B, D–F), in the Ly6C^{hi}/Ly6C^{med} ratio (Fig. 5 C) and in mean CD11b expression (Fig. 5 G–J). Only exception was the reduced CD11b expression in Ly6C^{lo} subset. This interesting observation suggests distinct effects of LCAT- and ApoA1-deficiency in circulating monocyte production.

Finally, to investigate if the reduced inflammatory phenotype of circulating monocytes from *Lcat*$^{-/-}$ mice results in less inflammatory tissue macrophages we performed gene expression analyses of common and M2 macrophage markers in liver tissue (Fig. 6 A). Livers of *Lcat*$^{-/-}$ mice showed a consistent trend towards downregulation of the common macrophage markers *F4/80*, *Cd68* and *Mcp1*, and an increase in the M2 marker *Mrc1*, suggestive for a reduced inflammatory phenotype of the liver. Moreover, peritoneal macrophages from *Lcat*$^{-/-}$ mice showed a markedly reduced LPS-induced TNFα response compared to macrophages from WT mice (Fig. 6 B). In line with the observed phenotypic differences between *Apoa1*$^{-/-}$ and *Lcat*$^{-/-}$ monocytes, peritoneal macrophages isolated from *Apoa1*$^{-/-}$ mice showed an increased LPS-induced TNFα response (Fig. 6 C). Collectively, these findings indicate that distinct mechanisms mediate the increased systemic inflammatory response observed in *Lcat*$^{-/-}$ and *Apoa1*$^{-/-}$ mice treated with LPS.

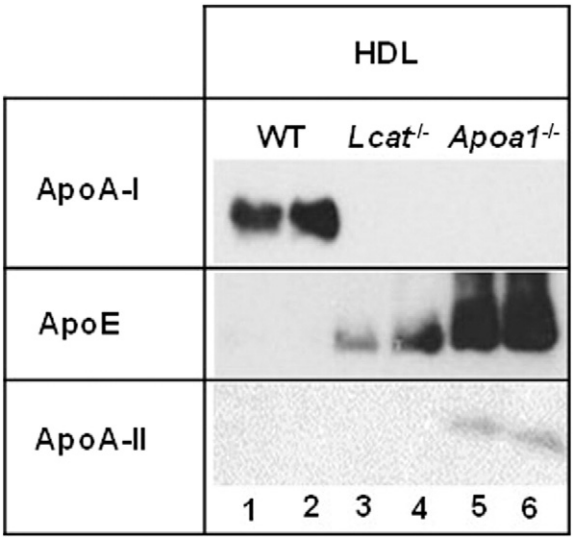

Fig. 1. *Lcat*$^{-/-}$ mice have mainly ApoE-containing HDL. Representative western blots for ApoA-I, ApoE and ApoA-II in HDL isolated from pooled serum of WT (lanes 1–2) and *Lcat*$^{-/-}$ (lanes 3–4) and *Apoa1*$^{-/-}$ mice (lanes 5–6). Total HDL (30 μg/μL protein) per genotype was loaded in two technical replicates.

4. Discussion

It is well established that mature HDL possesses potent anti-inflammatory properties and reduces LPS-induced inflammation *in vivo* [14] and *in vitro* [28]. Since most studies have focused on ApoA-I and mature HDL [14,15,29,30] the role of LCAT-mediated maturation of HDL on its anti-inflammatory potential remains largely unknown. To address this, we employed *Lcat*$^{-/-}$ mice that lack mature HDL, and compared them to *Apoa1*$^{-/-}$ mice which lack classical HDL, and WT animals. We report that LCAT-deficiency increases the systemic inflammatory response to LPS *in vivo*, impairs LPS-neutralizing capacity of circulating HDL and increases blood monocyte number. Importantly, this increased inflammatory response of *Lcat*$^{-/-}$ mice is reversed by reintroducing LCAT expression. In contrast to these pro-inflammatory effects, *Lcat*$^{-/-}$ mice have less pro-inflammatory blood monocytes and peritoneal macrophages. Finally, although both *Lcat*$^{-/-}$ and *Apoa1*$^{-/-}$ mice demonstrated a comparable inflammatory response *in vivo*, distinct differences were identified when comparing different aspects of the response *in vitro* (summarized in Table 4).

The increased LPS-induced pro-inflammatory response observed *in vivo* in *Lcat*$^{-/-}$ mice could be reproduced *in vitro* using whole blood or serum of these mice, indicating that alterations in serum components are responsible for these effects. Indeed our further analyses indicated that lack of mature HDL and the reduced LPS-neutralizing capacity of the circulating immature HDL contribute, at least in part, to the increased and prolonged inflammatory response of *Lcat*$^{-/-}$ mice *in vivo*. HDL reduces LPS-induced inflammation by binding LPS that is released in circulation, thereby inhibiting TLR4-mediated activation of macrophages [31] but also by facilitating the release of LPS that is already bound to macrophages [21,32]. Thus, absence of mature HDL in *Lcat*$^{-/-}$ mice logically contribute to reduced neutralization of LPS. In our studies, the immature HDL that is still present in *Lcat*$^{-/-}$ mice showed a reduced LPS-neutralizing capacity compared to HDL from WT mice, indicating that structural changes in HDL determine its neutralizing potential.

Our *in vitro* studies with serum also show that the LPS-induced TNFα response was more pronounced with low concentrations of LCAT-deficient and ApoA-I-deficient serum as compared to LPS alone. This intriguing observation was also seen previously for other 'pro-inflammatory' genotypes [33], though the mechanistic interpretation to this phenomenon is not clear at the present time. Another interesting observation is that lower concentrations of serum from *Lcat*$^{-/-}$ or *Apoa1*$^{-/-}$ mice have a higher pro-inflammatory effect compared to higher serum concentrations. Apparently, this is a known kinetic effect that relates to the thermodynamic equilibrium:[Free-LPS] + [serum factors] ⇔[bound LPS], with a K_d = [bound LPS] / ([Free-LPS] × [serum factors]). Bound LPS is neutralized, while free LPS is the active one. The more serum is added to the equilibrium (by increasing serum concentration) the more the equilibrium shifts to the right, that is more LPS is found in the bound (inactive) form, therefore more LPS is neutralized. In fact, high concentrations of serum factors, including for example the abundantly present albumin, may act like a sink for LPS *in vitro*.

We excluded the involvement of a direct bridging function of LCAT as addition of purified LCAT protein to LCAT-deficient HDL did not

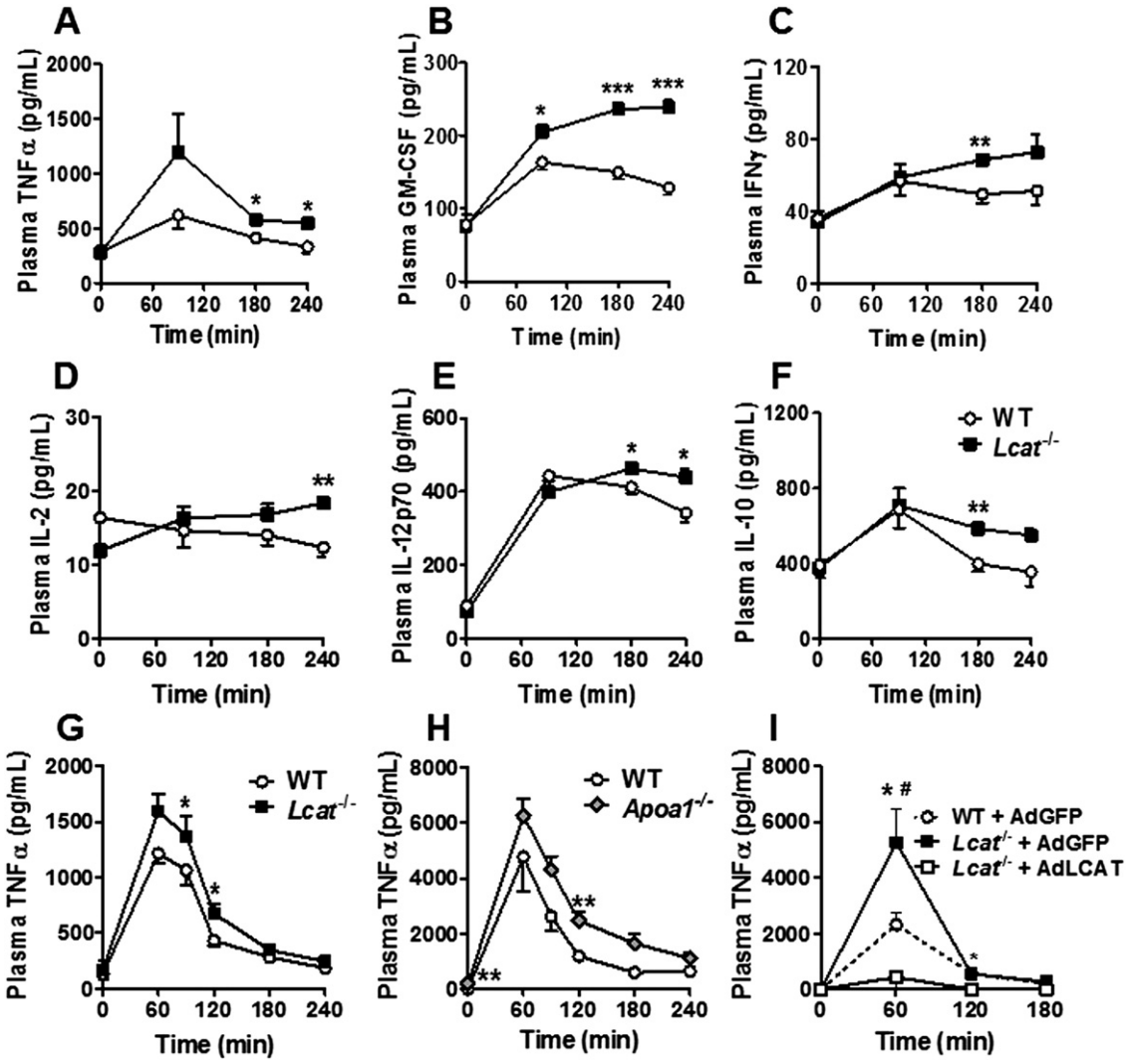

Fig. 2. LCAT-deficiency enhances the LPS-induced inflammatory response *in vivo*. LPS (100 μg/kg body weight) was intravenously injected in $Lcat^{-/-}$ and WT mice, and plasma TNFα (A), GM-CSF (B), IFNγ (C), IL-2 (D), IL-12p70 (E) and IL-10 (F) were determined at the indicated time points. Values represent mean ± SEM (n = 10; pooled per 2 mice). * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$ *vs* WT mice. LPS-induced plasma TNFα levels of individual $Lcat^{-/-}$ (G) and $Apoa1^{-/-}$ mice (H) were determined by ELISA. Values represent mean ± SEM (n = 6–10). * indicates $P < 0.05$ and ** indicates $P < 0.01$ *vs* WT mice. $Lcat^{-/-}$ mice were infected with 5×10^8 pfu of either AdGFP or AdLCAT and WT mice were infected with 5×10^8 pfu of AdGFP, and 3 days following infection all animals were intravenously injected with LPS and plasma TNFα levels were determined (I). Values represent mean ± SEM (n = 5–6). * indicates $P < 0.05$ *vs* $Lcat^{-/-}$ mice treated with AdLCAT and # indicates $P < 0.05$ *vs* WT mice treated with AdGFP.

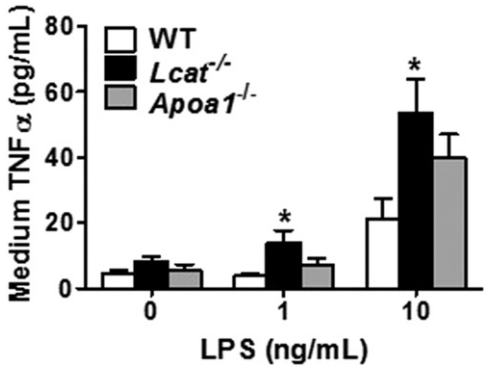

Fig. 3. LCAT-deficiency enhances the LPS-induced inflammatory response of whole blood *ex vivo*. Whole blood from $Lcat^{-/-}$, $Apoa1^{-/-}$ and WT mice was diluted in serum-free DMEM medium and subsequently incubated (18 h at 37 °C) without or with LPS (1–10 ng/mL). Data are representative for 2 experiments and expressed as mean ± SEM (n = 5). * indicates $P < 0.05$ *vs* WT blood.

improve its impaired neutralization capacity. Furthermore, we found no apparent LPS-binding motifs [26] in the amino acid sequence of LCAT. Thus, it appears that LCAT does not affect directly the LPS-induced TNFα-response. Particle geometry (discoidal *vs.* spherical) may be one other reason for the observed phenotypic differences. Another contributing factor to the observed lack of neutralizing capacity of HDL from $Lcat^{-/-}$ mice may be an altered protein cargo. Indeed, our analysis shows lack of ApoA-I in HDL of $Lcat^{-/-}$ mice. This is in agreement with dramatically lower ApoA-I plasma levels in $Lcat^{-/-}$ mice [34] and humans [35]. In addition, HDL of $Lcat^{-/-}$ mice contains significantly higher amounts of ApoE compared to WT mice.

Finally, we show that circulating monocytes are increased in $Lcat^{-/-}$ mice, a finding that could further explain the increased LPS-induced pro-inflammatory response. Unexpectedly however, the inflammatory potential of circulating monocytes and peritoneal macrophages was attenuated. Apparently, the increase in the number of circulating monocytes is explained by a marked increase in 'mildly-inflammatory' effector $Ly6C^{med}$ cells, whereas the number of 'pro-inflammatory' $Ly6C^{hi}$ monocytes was rather reduced. The reduced inflammatory status of the circulating monocytes and macrophages in $Lcat^{-/-}$ mice was further

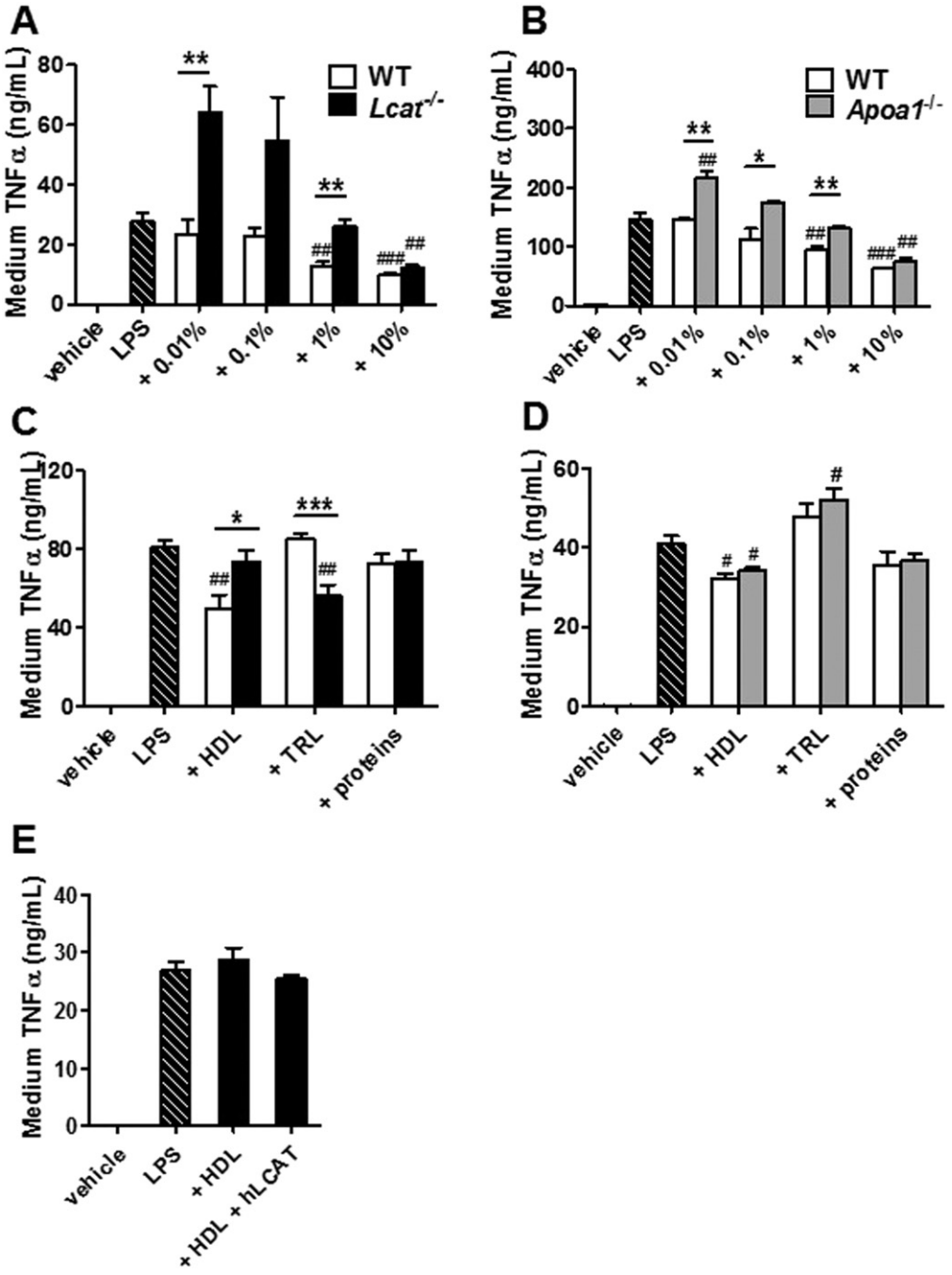

Fig. 4. LCAT-deficiency reduces the LPS-neutralizing capacity of serum and HDL *in vitro*. RAW 264.7 macrophages were incubated for 4 h without or with LPS (100 ng/mL) that was pre-incubated (30 min at 37 °C), with the indicated concentrations of serum from *Lcat*$^{-/-}$ (A) or *Apoa1*$^{-/-}$ (B) mice and compared to serum from WT mice. Similarly, RAW 264.7 macrophages were incubated without or with isolated HDL, TRL or lipid-free proteins (all 10 μg protein/mL) of WT, *Lcat*$^{-/-}$ (C) and *Apoa1*$^{-/-}$ (D) mice. RAW 264.7 macrophages were also challenged with LPS pre-incubated (100 ng/mL; 1 h at 37 °C) with HDL from *Lcat*$^{-/-}$ mice alone (0.01 μg/mL HDL-protein) or mixed with purified recombinant hLCAT protein (0.02 pg/mL, representing plasma LCAT levels in mice) (E). Values represent mean ± SEM (n = 3–4). # indicates $P < 0.05$, ## indicates $P < 0.01$ and ### indicates $P < 0.001$ *vs* LPS alone; * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$ *vs* WT serum/lipoproteins.

confirmed by the diminished CD11b expression in monocytes [36] and the reduced LPS-induced cytokine response of peritoneal macrophages. Recently, cholesterol efflux pathways have been highlighted as novel regulators of myelopoiesis [37]. Mice with defective cholesterol efflux (*i.e.* ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1)) showed increased hematopoietic stem cell proliferation, mobilization, and production of monocytes and neutrophils in bone marrow [38]. It is thus possible that reduced cholesterol efflux in *Lcat*$^{-/-}$ mice, caused either directly by the lack of LCAT-activity [39] or indirectly by the reduced ApoA-I content of immature HDL, stimulates myelopoiesis that in turn may account for the increased circulating monocyte. Our data thus indicate that LCAT-deficiency plays a very important role in monocyte production and inflammatory phenotype of these immune cells, though further studies are warranted.

To investigate the potential differences in the functional subsets (*i.e.* M1 and M2) of hepatic macrophages in *Lcat*$^{-/-}$ and WT mice, we used qRT-PCR to analyze the gene expression of some common and M2 macrophage markers. This analysis showed a significant upregulation of the *Mrc1* gene which encodes for the mannose receptor, present on the surface of macrophages and immature dendritic cells. MRC1 is implicated in phagocytosis, antigen presentation, intracellular signaling, resolution of inflammation,

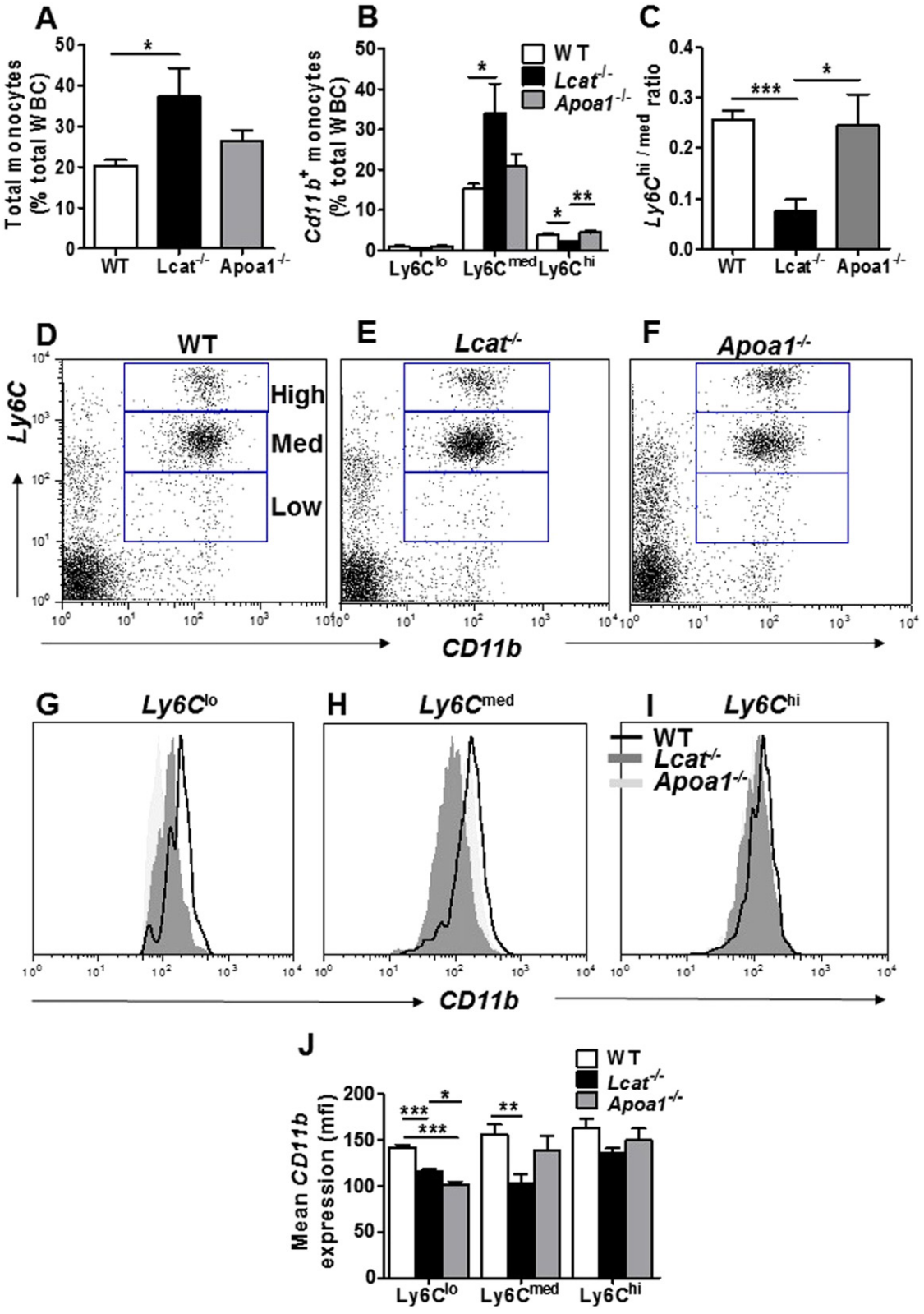

Fig. 5. LCAT-deficiency associates with an increased number but reduced inflammatory phenotype of circulating monocytes. Total circulating blood $Cd11b^{+}/Ly6C^{+}$ monocytes were determined by flow cytometry in $Lcat^{-/-}$, $Apoa1^{-/-}$ and WT mice (A). $Ly6C^{lo}$, $Ly6C^{med}$ and $Ly6C^{hi}$ within $CD11b^{+}$ monocytes were gated and expressed as % of total white blood cells (WBC; B). $Ly6C^{hi/med}$ ratio was calculated as a marker of "anti-inflammatory" phenotype (C). Representative dot plots showing the monocyte subpopulations are shown (D–F). Representative histograms with Cd11b expression (Mean Fluorescence Intensity) are shown (G-I) as well as mean Cd11b expression of the monocytes subpopulations in the different genotypes (J). Values represent mean ± SEM (n = 5). * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ between groups.

and clearance of glycoprotein hormones [40]. One of its most important roles is in the resolution of inflammation, since it ensures the removal of inflammatory agents (high-mannose oligosaccharides) from the circulation at the late phases of inflammatory responses [41]. Overall this analysis suggests a more anti-inflammatory and possibly regulatory phenotype for hepatic macrophages in $Lcat^{-/-}$ mice.

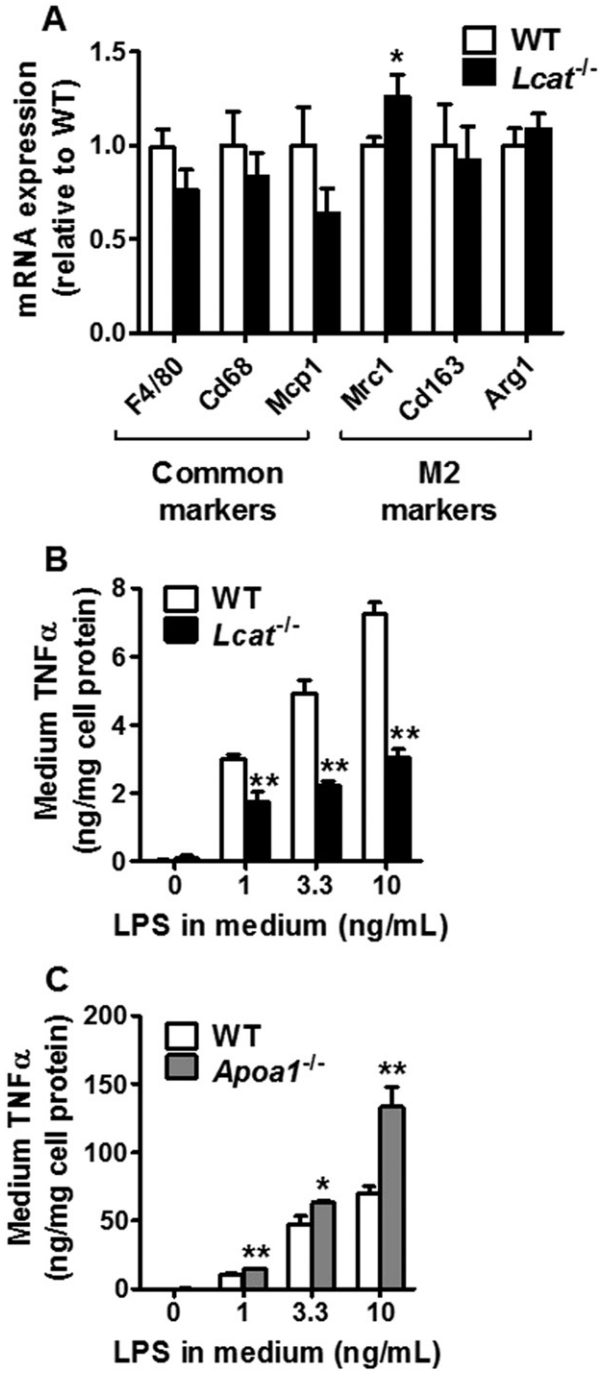

Fig. 6. LCAT-deficiency associates with reduced hepatic inflammatory state and dampened peritoneal macrophage responsiveness to LPS. Relative mRNA expression levels of general and M2 macrophage markers in livers of *Lcat*$^{-/-}$ mice as compared to WT controls (A). Values represent mean ± SEM (n = 8). * indicates $P < 0.05$ between groups. Thioglycollate-stimulated peritoneal macrophages from WT and *Lcat*$^{-/-}$ (B) as well as from WT and *Apoa1*$^{-/-}$ mice (C) were isolated after peritoneal lavage, washed, pooled and plated (8×10^5 cells/well in 24 well plates). The next day macrophages were incubated (4 h, 37 °C) without or with LPS (1–10 ng/mL) in DMEM. Data are representative of 2 experiments and expressed as ng/mg cell protein and represent mean ± SEM (n = 4). * indicates $P < 0.05$, and ** indicates $P < 0.01$ *vs* WT group.

5. Conclusions

Taken together, this study extends our current understanding on the anti-inflammatory function of HDL showing that qualitative changes in the lipoprotein particle may exert distinct effects on its anti-inflammatory potential. We show that LCAT-deficiency triggers both pro- and anti-inflammatory effects, however the net systemic response of *Lcat*$^{-/-}$ mice to LPS challenge is pro-inflammatory. Although ApoA-I-deficient mice display a similar LPS-induced response *in vivo*, the underlying mechanisms are mostly distinct. We conclude that LCAT-mediated maturation of HDL plays a crucial role in neutralizing circulating LPS and endotoxemia, but also an unexpected role in determining the number and phenotype of monocytes and macrophages.

Transparency Document

The Transparency Document associated with this article can be found, in the online version.

Conflict of interest

The authors have no financial conflicting interests to disclose.

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