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

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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.



## 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.

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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 $2 \times 10^{10}$ pfu/mL were obtained for each virus preparation. *Lcat*$^{-/-}$ mice were injected with either AdGFP or AdLCAT adenovirus at a low dose of $5 \times 10^{8}$ 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