Lipoproteins in Innate Immunity
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CHAPTER I

General Introduction
Chapter 1
General introduction

1. Infection and sepsis
Sepsis is still one of the major causes of death in intensive care units. In the USA, it ranks 13th as cause of death (1) and is on the increase despite the development of new supportive therapies. It is estimated that the number of cases per year rose from 3000 in 1990 to 4000 in 1996 in the Netherlands (2). Sepsis can be defined as the pathophysiological alterations and clinical consequences of the presence of microorganisms or their products in the bloodstream or tissues (3). A full panel of microorganisms, including Gram-negative and Gram-positive bacteria, fungi, pathogenic viruses and rickettsia can trigger the pathophysiological cascade leading to sepsis (4). Secondary symptoms of the infection are temperature alterations, leukocytosis, hypoperfusion of tissues and cell death which may lead to organ failure and septic shock frequently culminating in mortality (1). The latter symptoms can be diagnosed as the systemic inflammatory response syndrome (SIRS) (1). Septic shock is diagnosed when the patient becomes hypotensive as a consequence of the host immune response.

1.1 Structure of endotoxin (LPS and LTA)
The cell wall of Gram-negative bacteria consists of three layers: an inner membrane, a peptidoglycan layer and an outer membrane. The major component of the outer membrane is a glycolipid termed lipopolysaccharide (LPS) that is composed of a polysaccharide chain and a lipid moiety called lipid A (5). The lipid part is embedded in the outer membrane of Gram-negative bacteria, whereas the polysaccharide part protrudes into the environment. The polysaccharide part consists of an O-specific side-chain or O-antigen and “core” sugars. The core is divided in the inner core, which is linked to lipid A, and the outer core, which is linked to the O-specific chain (Fig. 1). The highly conserved lipid-A part of LPS is highly conserved among Gram-negative bacteria strains. A common feature of the lipid A part is a β. 1-6 linked disaccharide of glucosamine phosphorylated at the position 1’ and 4’ to which 4 to 6 fatty acids are attached (5).
Lipid A is directly linked via the 6' position to the 3-deoxy-D-manno-octulosonic acid (KDO) of the inner core. Besides KDO, the inner core also contains heptose sugars. Both KDO and heptose are quite uncommon sugars and are specific for Gram-negative bacteria. In contrast to the inner core, the outer core is composed of common sugars, e.g. D-glucose, D-galactose and N-acetyl-D-glucosamine. Most LPS forms contain a repeating oligosaccharide chain attached to one of the glucose molecules of the outer core. The O antigen is, in contrast to the core segment and lipid-A structure, highly variable and it is this part of the LPS that accounts for specific immune reactions in the host.

The complete LPS structure as described above is not absolutely necessary for bacteria to survive. Some strains lack the O-specific side-chain and these are designated rough mutants, because of the visual appearance of rough colony morphology. Bacterial strains with complete LPS molecules form smooth colonies and this LPS is referred to as smooth. The truncated LPS structures (“chemotypes”) of the rough mutants are classified Ra to Re. (6), (7). Although the O-antigen is not strictly required for survival, it forms a shield that prevents entry of complement factors and phagocytes, and thereby extends the bacterial life span in vivo. (5).

Lipoteichoic acid (LTA) has been proposed as putative Gram-positive immuno-stimulatory membrane component (8). LTA and peptidoglycans are the major cell wall components of Gram-positive bacteria (9) and are members of structurally related macro-amphiphiles that consist of glycolipids. LTAs are composed of a hydrophobic diacylglycerol membrane anchor and a hydrophilic head-group extending towards the bacterial surface (10). LTA is present in most Gram-positive bacteria and may initiate septic shock (9).
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1.2 Acute phase response

The acute phase response (APR) is characterized by dramatic changes in the concentrations of specific plasma proteins, which are assumed to protect the host from further injury, and facilitate the repair process (11). The activation of the APR via innate immunity is dependent on recognition of pathogen-associated molecular patterns (PAMP), which enable the host immune system to rapidly recognize bacterial components such as LPS, peptidoglycan, dsRNA, bacterial DNA, and flagellin (12). LPS or LTA are presented to the Toll-like-4 (TLR-4) or TLR-2 receptors, respectively on the surface of monocytes, macrophages and dendritic cells (13), (14), thereby causing induction of MAP kinase pathways and nuclear factor kappa B (NFκB), which in turn regulate gene expression of the pro-inflammatory cytokines and chemokines (15). Tumor necrosis factor alpha (TNFα) (16), interleukin-1β (IL-1β) and interferon-γ (IF-γ) (17), are the first cytokines that are induced followed by interleukin-6 (IL-6) (18), (19) (B-lymphocytes activator) and chemokines, such as IL-8 (20) (chemo-attractant). The release of the anti-inflammatory cytokine interleukin-10 (IL-10) (21), inhibitors of TNF-α (soluble surface receptor TNF-α) (22), and interleukin-1α (IL-1α) (IL-1 receptor antagonist) (23) is thought to balance the inflammatory process.

Cytokine production causes pleiotropic effects in the host such as activation of neutrophils (24), (25), and adherence of neutrophils to endothelial cells (26), apoptosis (27), activation of the extrinsic pathway of coagulation (28), (29) and the production of acute-phase proteins (30). Further, the concentrations of positive acute-phase proteins, C-reactive protein (CRP) (31), serum amyloid A (32), (33), fibrinogen (34) and haptoglobin (35) increase during the APR, whereas plasma concentrations of negative acute-phase proteins such as albumin, Factor XII, and transferrin decrease (36). CRP, which is mainly produced in the liver, is a member of the pentraxin family, which consists of proteins with a characteristic pentameric organization of identical subunits (31). This protein is widely used as a marker of an ongoing inflammatory process. It recognizes foreign pathogens as well as phospholipid constituents of damaged cells (37), and following binding to its ligands can activate the complement system. Interestingly, CRP has been found in association with lipoproteins in vitro (38), (39).

Apart from the changes discussed, the APR has a drastic impact on lipid metabolism, which will be discussed in more detail in section 4.
2. **Lipoprotein metabolism**

2.1. **Lipoproteins**

Lipoproteins are primarily responsible for the transport of lipids in the circulation. These spherical particles are composed of a neutral lipid core (containing cholesteryl esters and triglycerides), a polar phospholipid/cholesterol monolayer, and amphipathic apolipoproteins and proteins, which are not involved in lipid metabolism but enable lipoproteins to function as carrier particles (40). Lipoproteins vary in size, density, electrophoretic mobility, composition, function and metabolism. Depending on the isolation technique lipoproteins can be divided in several subclasses. Using the classical differential ultra centrifugation based on the hydrated density, five major lipoprotein classes can be separated: Chylomicrons (CM, $d < 0.94$ g/ml), very low density lipoproteins (VLDL, $0.94 < d < 1.006$ g/ml), intermediate density lipoproteins (IDL, $1.006 < d < 1.019$), low-density lipoproteins (LDL, $1.019 < d < 1.063$), and high density lipoproteins (HDL, $1.063 < d < 1.21$) (41). An additional lipoprotein class, lipoprotein (a) (LP-a), contains a hydrophilic apolipoprotein (a) covalently coupled via a disulphide bridge to apo B100, the major apolipoprotein of LDL (42).

Cholesterol plays an important role in the regulation of the fluidity and barrier function of cell membranes and is required for the endogenous synthesis of bile acids and steroid hormones. Triglycerides are used as an energy source for cardiac and smooth muscle cells, and are stored in adipose tissue. Each lipoprotein class contains specific apolipoproteins that are essential for preservation of the integrity of the lipoprotein particles and serve as ligands for lipoprotein receptors and function as cofactors or inhibitors of enzymes such as lipoprotein lipase (LPL) and hepatic lipase (HL). An overview of the main constituents of the lipoproteins is summarized in Table 1.

2.2 **Lipoprotein metabolic routes**

Lipids are delivered to and removed from peripheral tissues by three main metabolic routes. The exogenous pathway (Figure 2 section A) regulates the uptake of dietary lipids by the body, the endogenous pathway (Figure 2 section B) delivers lipids throughout the body, and the reverse cholesterol pathway (figure 2 section C) regulates peripheral cholesterol transport towards the liver.
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Table 1. The physical properties and composition of human plasma lipoproteins.

<table>
<thead>
<tr>
<th></th>
<th>Chylomicrons</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mw (Da)</td>
<td>&gt;5 MDa</td>
<td>5 MDa</td>
<td>1 MDa</td>
<td>300,000</td>
</tr>
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<td>Diameter (nm)</td>
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<td>30-80</td>
<td>18-25</td>
<td>5-12</td>
</tr>
<tr>
<td>Mobility (position)</td>
<td>origin</td>
<td>Pre-β</td>
<td>Pre-β</td>
<td>Pre-α</td>
</tr>
<tr>
<td>Protein (g%)</td>
<td>1-2</td>
<td>6-10</td>
<td>21</td>
<td>45-55</td>
</tr>
<tr>
<td>Triglycerides (g%)</td>
<td>88</td>
<td>56</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Phospholipids (g%)</td>
<td>8</td>
<td>20</td>
<td>28</td>
<td>45</td>
</tr>
<tr>
<td>Esterified chol. (g%)</td>
<td>3</td>
<td>15</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td>Free cholesterol (g%)</td>
<td>1</td>
<td>8</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Apolipoproteins</td>
<td>A/I/AIV,B48.</td>
<td>B100</td>
<td>B100</td>
<td>A/I/AIV</td>
</tr>
<tr>
<td></td>
<td>Cl,Ch,Chl</td>
<td>Cl,Ch,Chl</td>
<td>Cl,Ch,Chl</td>
<td></td>
</tr>
</tbody>
</table>

2.2.1 Exogenous pathway

Chylomicrons (CM) are synthesized by the intestine and secreted into the mesenteric lymph from which they enter the general circulation (43). In the circulation, chylomicrons recruit apolipoproteins, apo C, apo A-I and apo E from other circulating lipoproteins. Apo C serves as a cofactor for LPL for the hydrolysis of triglycerides. As a consequence of hydrolysis, CM become smaller and an excess of phospholipids with associated apolipoproteins is shed from the particle and can be incorporated into HDL. The residual particles are called CM-remnants and are taken via the hepatic receptors (LDL receptor and the LDL receptor related protein (LRP)), which recognize apo E still present on the CM-remnant (44).

2.2.2 Endogenous pathway

VLDL, that contains apo B100 and small amount of apo E and apo C, is synthesized in the liver and secreted into the circulation (45). Within the periphery, LPL hydrolyses triglycerides in the VLDL core, which results in the formation of intermediate-density lipoprotein (IDL) or VLDL remnant particles. These IDL particles are further converted by hepatic lipase activity and are taken up by the liver via the apo E receptor, eventually yielding LDL (46). LDL is rich
in cholesterol esters and contains the ligand protein apo B100 that is necessary for LDL receptor mediated uptake in the liver.

2.2.3. Reverse cholesterol pathway

Cholesterol in peripheral tissues may be transported back to the liver, mainly by HDL. Nascent or pre-β HDL is synthesized in the liver and in the intestine from chylomicron remnant particles. This nascent HDL takes up
cholesterol from extra hepatic tissues via the ATP Binding Cassette A-1 (ABC-A1) receptor (47) and transports it to the liver where HDL is bound by the scavenger receptor SR-B1 (48). Cholesterol is converted to bile acids, which are excreted into the intestinal tract. This transport of cholesterol is dependent on several lipid transport proteins, such as lecithin cholesterol acyl transferase (LCAT), cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP). LCAT is needed for the esterification of free cholesterol into cholesterol esters during the conversion of pre-β HDL into mature HDL. CETP is involved in exchange of cholesterol and triglycerides from HDL to LDL/VLDL, whereas PLTP is involved in the exchange of phospholipids between lipoproteins. The properties of CETP and PLTP are discussed in more detail below.

2.3 The LPS-binding/lipid transfer protein family

The four lipopolysaccharide-binding/lipid transfer proteins are bactericidal/permeability-increasing protein (BPI), lipopolysaccharide-binding protein (LBP), phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP). These proteins have 20-26% sequence homology, share structural and functional properties and together form the lipopolysaccharide-binding/lipid transfer protein family (49), (50).

BPI, a cationic protein has a calculated molecular weight of 51 kDa, probably as a consequence of glycosylation the observed SDS-PAGE molecular weight is 58 kDa. BPI seems to be tightly associated with the membrane of the azurophilic granules of polymorphonuclear leukocytes (51). It is capable of binding to the bacterial membrane where it causes growth cessation and lysis following phagocytosis of bacteria (52). In 1997, the crystal structure of BPI was elucidated (53), which revealed a boomerang-shaped molecule (Fig. 3). The two main domains are barrel-shaped and are connected by a central beta sheet. Each barrel was found to contain a pocket with a single molecule of phosphatidylcholine. Both pockets are predicted to be involved in the binding of a single LPS molecules (as indicated in Fig. 3).
CETP is a hydrophobic plasma glycoprotein with a molecular weight that varies from 66 to 74 kDa after polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) due to differences in glycosylation. CETP is mainly associated with HDL particles and is responsible for all neutral lipid exchange activity in plasma. It facilitates the transport of cholesteryl esters to triglyceride rich particles (VLDL, LDL and chylomicrons) in exchange for triglycerides (54). Moreover, CETP is responsible for phospholipid transfer between lipoproteins by forming a ternary complex between the protein and lipoproteins thereby causing fusion of the lipoprotein particles (55). Further, CETP mediates the selective uptake of cholesteryl esters by human adipose tissue (56) and is directly involved in the formation of pre-beta HDL. The CETP mass and activities are decreased upon LPS administration in vivo (57), which has major effects on HDL levels, and may represent a protective adaptive response for the preservation of the HDL population (58).

PLTP is mainly associated with HDL and has an apparent molecular weight of 69 – 81 kDa depending its state of glycosylation (59), and purified PLTP proteolytic 51 kDa fragments have been observed. Spontaneous diffusion of phospholipids is too slow to be physiologically important and PLTP has two major functions: It affects in HDL metabolism by regulating the transfer of phospholipids from cells membranes towards HDL (60) and in the modulation of HDL composition (61) and size (62). These properties indicate that PLTP plays
Fig. 4. LPS routes of transport in blood. (1) Stimulation and (2 and 3) inhibition of the inflammatory response and (4) clearing of lipoprotein associated LPS via the liver. CM: Chylomicrons, LDL: low-density lipoprotein, VLDL: very low-density lipoprotein, PLTP: phospholipid transfer protein, CETP: Cholesteryl ester transfer protein.

an important role in maintaining normal HDL levels in plasma (63). PLTP has also been reported to neutralize and transport LPS from vesicles to reconstituted HDL (rHDL) (64). However, PLTP is unable to transfer LPS to CD14 and seems not to play an important role in PLTP-mediated LPS transport to cells (64).

LBP is a glycosylated plasma protein with an apparent molecular weight of 60 kDa (Beamer). LBP is predominantly synthesized by the liver, but during inflammatory stimuli, additional expression is found in the lung, kidney and heart (65). The function and role of LBP is discussed in more detail below.

3. **Endotoxin binding by lipoproteins**

Lipoproteins bind and inactivate bacterial endotoxins *in vitro* and *in vivo* (66), (67), (68), and this function is an integral part of the initial host defense mechanism (69). Chylomicrons, VLDL (70), (71), (72), LDL (67), (70) and HDL (70), (73), (74) are all capable of binding endotoxin, and as a result most LPS in blood is found in association with lipoproteins with less than 5% being bound to other plasma proteins (73), (75) such as albumin, transferrin or the immunoglobulines IgG and IgM (73). Importantly, binding to the latter proteins, in contrast to lipoprotein binding, does not inactivate LPS bioactivity (70). Of all lipoproteins, HDL appears to have the highest binding capacity for endotoxin (75), (73), (76).
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LBP critically orchestrates the LPS-induced inflammatory response by mediating LPS transfer to membrane-bound CD14 and soluble CD14 (Fig. 4). CD14 facilitates transfer of LPS to the Toll-like receptor 4, which is responsible for LPS recognition. LBP is associated with HDL (77) and during the acute phase response may also be found on LDL (78), (79). LBP is capable of mediating uptake of LPS or LTA by these lipoproteins, which results in the attenuation of the acute phase response. Lipoproteins such as HDL may also scavenge LPS from cell membranes (80), (81).

Following binding to triglyceride rich lipoproteins such as chylomicrons and VLDL (82) LPS is presented to hepatocytes (71) and cleared via the bile, thus avoiding activation of Kupffer cells (the hepatic macrophages) (66), (83). In rodents, a low plasma triglyceride concentration is associated with increased LPS-induced mortality, which can be reversed by lipoprotein substitution (82). In humans, induction of hypertriglyceridemia by treatment with Intralipid (a fat emulsion, which resembles triglyceride-rich lipoproteins) did not attenuate the host response to LPS *in vivo* (84), whereas treatment with a synthetic HDL particle (reconstituted HDL or rHDL), which contains apo A-I, cholesterol and phosphatidylcholine, cytokine production in whole blood (*ex vivo*) (74) and during experimental endotoxemia (*in vivo*) (85), (86) was prevented.

4. **Changes in lipoprotein metabolism during the acute phase response**

Plasma lipid levels are determined by a balance between synthesis and clearing rates. During the acute phase reaction, drastic alterations in the homeostasis of lipid metabolism occur. Plasma cholesterol levels are invariably decreased, independent of the type of infection, but the severity of decrease is related to the extent of the inflammatory reaction (87). Plasma triglyceride levels may increase, remain constant, or decrease depending on the acute phase condition (88). Hepatic triglyceride production is however always increased (89) due to an increased availability of free fatty acids released by stimulated lipolysis in the peripheral and adipose tissues, or by *de novo* synthesis in the liver. These processes are regulated by the cytokines (90), TNF-α, IL-1 and the interferons α, β, and γ, or in the case of *de novo* lipoprotein synthesis by TNF-α and β. IL-1, IL-6 and interferon-α (91), (92).
4.1. Changes in VLDL

The increase in plasma triglycerides is mainly caused by an increase in VLDL (93) (94). Low doses of LPS and high doses of LTA induce cytokine production and can mimic the symptoms of Gram-negative or Gram-positive infections in animals and humans. LPS and LTA cause an increase in VLDL levels by decreasing VLDL clearance and increasing VLDL synthesis (95), (96). High doses of LPS do not affect VLDL synthesis, but do decrease LPL activity (88).

Besides the alterations in VLDL and TG composition during infection, newly synthesized VLDL particles are enriched in sphingolipids (97), (98). It has been described that sphingomyelin causes an impairment of VLDL clearance that may probably be compensatory mechanism for restoration of lipid homeostasis, but which may result in the accumulation of pro-atherogenic remnant particles (99).

4.2. Changes in LDL

Upon LPS or LTA administration, the TG and cholesterol content of LDL increases in non-primates (100), (97), and the appearance of a subclass of LDL, known as small dense LDL, has been reported (101). The current hypothesis is that small dense LDL is atherogenic because it is more susceptible to oxidation and it is able to penetrate the endothelium and bind to intima proteoglycans, which results in trapping in the arterial wall (102). Small dense LDL particles are enriched in sphingolipids, including sphingomyelin and ceramide (98). The enzyme serine palmyoyl-transferase is upregulated during the acute phase, which leads to a higher production of sphingomyelin. Since sphingomyelin serves as a pool for conversion to ceramide, LDL ceramide also levels increase during sepsis (103), (104). In addition, the glucosylceramide content of lipoproteins rises due to an increased activity of glucosylceramide-synthase following LPS administration (105).

The levels of the pro-inflammatory phospholipid platelet-activating factor (PAF) are increased during sepsis (106) and cause several biological effects such as activation of inflammatory cells, increased vascular permeability and hypotension. The acute phase-proteins such as platelet activating factor acyl-hydrolase (PAF-AH) (43), LDL and HDL constituents, and secretory non-pancreatic phospholipase A2 (sPLA2) (107) are all increased during sepsis. PAF-AH hydrolyses PAF, which is a protective mechanism but also results in the
hydrolysis of phosphatidylcholine into the more atherogenic lyso-phosphatidylcholine (44). Finally sPLA₂, associated with small dense LDL (108) hydrolyses phosphatidylethanolamine and cleaves polyunsaturated fatty acids from the sn-2 position (109). These fatty acids are very susceptible to peroxidative damage and may lead to an increase in the atherogenic potential of LDL.

4.3. Changes in HDL

Irrespective of the causal organism, infection is associated with large decreases in the HDL cholesterol and apo A-I content (this thesis), (110), (100), (111) and in the enrichment of free cholesterol, triglycerides and sphingolipids (97), (100), (112). Apart from the lipid alterations in HDL, the protein composition markedly changes during the course of sepsis. Increased serum amyloid A (SAA), apo J, sPLA₂, and ceruloplasmin concentrations, and decreased apo A-I, paraoxanase, LCAT and PLTP mass levels have been observed (2), (113). These altered HDL particles that circulate during infection and inflammation are known as “acute phase HDL” (114). Of particular importance is the association of SAA with HDL, which displaces apo A-I (115), (116), (117). The presence of SAA on HDL reduces the affinity of HDL for hepatocytes by 2 fold but increases the affinity for macrophages some 3 to 4 fold (118). This causes a redirection of the HDL clearing route from the liver towards macrophages (119) and has been suggested to lead to a functional change of the originally anti-atherogenic to a pro-atherogenic HDL particle as a consequence of the resulting delivery of cholesterol to peripheral tissues (117).
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5. Outline of this thesis

The aim of this thesis was to examine the importance of lipoproteins in innate immunity. In chapter 2 and 3 the LPS and LTA sequestering capacity of lipoproteins ex vivo was investigated, using very mild separation- and preparative methods. In chapter 4, the differences in LPS sequestering of HDL subtypes were examined. Further, in chapter 5 the LPS binding capacity of lipoproteins ex vivo in plasma and lymph obtained from patients with SIRS and multi-organ failure was studied. Additionally in chapter 6, the capacity of PLTP and LBP to redistribute HDL associated LPS between LDL and VLDL in vitro was studied.

Treatment of sepsis is often limited to administration of antibiotics, fluids, vasopressors and cardiac, pulmonary and metabolic support. In the light of the LPS neutralizing properties, the survival of rats upon LPS administration and treatment with reconstituted HDL (a synthetic HDL) with or without bile-duct ligation was investigated. This is presented in chapter 7. In chapter 8, the overall lipoprotein, apolipoprotein and phospholipid changes during experimental endotoxemia and during clinical sepsis in primates with time are presented. Furthermore, the lipoprotein alterations during rHDL treatment following LPS administration in human volunteers were investigated.

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