EFFECT OF PLASMA PHOSPHOLIPID TRANSFER PROTEIN DEFICIENCY ON LEthal ENDOTOXEMIA IN MICE*

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Lipopolysaccharides (LPS) are components of Gram-negative bacteria. The cellular response from the host to LPS is mediated through stepwise interactions involving the lipopolysaccharide binding protein (LBP), CD14, and MD-2 which produces the rearrangement of TLR4. In addition to LBP, the lipid transfer/lipopolysaccharide binding protein (LT/LBP) gene family includes the phospholipid transfer protein (PLTP). Here we show that the intravascular redistribution of LPS from the plasma lipoprotein-free fraction towards circulating lipoproteins is delayed in PLTP-deficient mice. In agreement with earlier in vitro studies which predicted the neutralization of the endotoxic properties of LPS when associated with lipoproteins, significant increases in the plasma concentration of pro-inflammatory cytokines were found in PLTP-deficient as compared to wild-type mice. Similar inflammatory damage occurred in tissues from wild-type and PLTP-deficient mice 24 hours after one single intraperitoneal injection of LPS, however with a more severe accumulation of red blood cells in glomeruli of LPS-injected PLTP-deficient mice. Complementary ex vivo experiments on isolated splenocytes from wild-type and PLTP-deficient mice came in further support of the ability of cell-derived PLTP to prevent LPS-mediated inflammation and cytotoxicity when combined with lipoprotein acceptors. Finally, PLTP deficiency in mice led to a significant increase in LPS-induced mortality. It is concluded that increasing circulating levels of PLTP may constitute a new and promising strategy in preventing endotoxic shock.

Lipopolysaccharides (LPS) or endotoxins are major constituents of the outer membrane of Gram-negative bacteria (1). The presence of LPS in the bloodstream activates the innate immune system through LPS recognition and production of pro-inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor-alpha (TNFα) (2-4). Abundant LPS-mediated secretion of pro-inflammatory cytokines leads to uncontrolled inflammatory response, known as endotoxic shock. It is characterized by fever, disseminated intravascular coagulation, hypotension, circulatory collapse, multiple organ failure, and death (2-4).

In vivo, LPS is targeted to inflammatory cells through a transfer reaction initiated by the lipopolysaccharide binding protein (LBP) (5-9). LPS monomer is delivered by LBP to the glycosphingolipid insitol-linked receptor CD14 (cluster of differentiation-14) which exists either as a membrane anchored (mCD14) or a soluble (sCD14) protein (10-12). CD14 is required to transfer LPS to MD-2. Finally, the formation of trimeric LPS:MD-2:TLR4 complexes initiate the Toll-like receptor 4 (TLR4)-mediated signal transduction pathway that leads to the increased release of pro-inflammatory cytokines (13-16). If instead of binding to LBP, LPS binds to circulating lipoproteins, including chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL), its activity is neutralized. Compared to LPS in the plasma lipoprotein-free fraction (LFF), LPS-lipoprotein complexes have been shown to be less pyrogenic, less able to activate complement, and less active in inducing the...
release of cytokines (IL-1, IL-6, and TNFα), resulting in fewer deaths in lipoprotein-treated animals (17-23). In addition, LPS-lipoprotein complexes are detoxified through the binding of lipoproteins to their cell surface receptors, in particular hepatocytes resulting in increased biliary excretion of endotoxins (21, 24, 25). In humans, both high circulating levels of plasma HDL and infusion of recombinant HDL have been shown to be associated with significant reductions in the production of pro-inflammatory cytokines, in cell activation, and in the clinical symptoms of sepsis (26-28).

In addition to LBP, the LT/LBP (lipid transfer/lipopolysaccharide-binding protein) family includes the bactericidal permeability increasing protein (BPI), the cholesteryl ester transfer protein (CETP), and the phospholipid transfer protein (PLTP). PLTP was first identified for its implication in lipoprotein metabolism and the development of atherosclerosis (29-31). Whereas in vitro studies have shown that LBP and PLTP can extract LPS from Gram-negative bacterial membranes (32), the relative in vivo contribution of these proteins to either triggering or blocking the CD14-mediated activation of human monocytes by LPS is unclear (5, 7, 33-35). Recombinant human LBP can induce both the binding of LPS to human monocytes and LPS-mediated secretion of TNFα (36), but no significant differences in the clearance of LPS (6) or in TNFα levels (37) were observed in the plasma of wild-type (WT) and LBP-deficient mice injected with LPS. In the case of PLTP, only in vitro data showing its LPS transfer activity have yet been reported (32, 33, 35), and the possibility that alterations in PLTP expression in vivo may modify plasma LPS distribution and clearance, cytokine production and mortality following endotoxic shock has never been investigated. In the present study, the physiopathological impact of PLTP on LPS metabolism and endotoxic shock was evaluated through the comparison of PLTP-knockout out mice with WT mice, which combine high plasma PLTP activity (38) and a remarkable resistance to LPS-mediated endotoxic shock (39).

**EXPERIMENTAL PROCEDURES**

**Animals**- PLTP-deficient mice were previously generated by Dr Jiang XC and colleagues (40). Briefly, they were obtained by replacing exon 2 (containing the translation start site) with a neo gene (40). Animals were on an homogenous C57BL/6 background (eight backcrosses). PLTP+/homozygous mice, PLTP+-/- heterozygous mice, and WT/non-targeted C57BL/6 mice (4 to 6 months old) were fed a standard chow diet (A03 diet, Safe, Augy, France). Animals had free access to water and food. All experiments involving animals were performed in accordance with institutional guidelines and approved by the University of Burgundy’s Ethics Committee on the Use of Laboratory Animals (protocol number 2606).

**LPS Injection and Blood Sampling**- All materials were apyrogen or depyrogenated by steam autoclaving before use, and all the reagents used were of ‘endotoxin-free’ grade. As assessed by using either the LAL assay or the GC-MS 3-hydroxymyristic acid quantitation (see below), only trace amounts of LPS were detected in experimental media which were not supplemented with exogenous LPS. LPS (Escherichia coli serotype 055:B5, Sigma-Aldrich, St. Louis, MO, USA) was suspended in endotoxin-free sodium chloride and vigorously mixed for 15 min before use. The LPS was injected intraperitoneally into mice (5 mg/kg or 0.5 mg/kg of body weight; single doses). Blood was then collected at the indicated times on endotoxin-free sodium citrate (1/9, v/v) (Sigma) via caudal or retroorbital puncture. Plasma was obtained by blood centrifugation (10 min, 2000 g at 4°C), and lipoproteins were further isolated from the plasma by sequential ultracentrifugation as previously described (39). The d>1.21 g/L fraction constituted the LFF. All samples were stored at -80°C until further analysis.

**Phospholipid transfer activity**- PLTP activity was measured as previously described (41), using a commercially available fluorescence activity assay (Roar Biomedical Inc, New York, NY, USA) according to the manufacturer’s instructions. This fluorimetric assay measures the transfer (unquenching) of fluorescent phosphatidylcholine from donor to acceptor synthetic liposomes. Phospholipid transfer rates were calculated as the initial slope of the phospholipid transfer curve. They were expressed as increase in fluorescence per second (Arbitrary Units per second, AU/s).
PCR were conducted by using a mixture of 3 primers: 5’-GCAGCGCATCGCCTTCTATC-3’ (Neo3), 5’-AAAGGCTGCCGACCAGCG-3’ (66AM), and 5’-TGGTCATGCATCTAGAACGGAGT-3’ (58BM). Amplification products were analysed by 2.0% agarose gel electrophoresis containing ethidium bromide, and molecular weights were determined as compared to the 100bp DNA Ladder calibration kit (Invitrogen, UK). One single, 580bp band is obtained in WT mice, one 580bp band plus one 680bp band are obtained in PLTP+/− heterozygotes, and one single, 680bp band is obtained in PLTP−/− homozygotes.

LPS Quantification- LPS in total serum, bile or isolated lipoprotein fractions was assayed by using either a biological activity assay (Limulus Amebocyte Lysate (LAL) kit - QCL-1000 chromogenic assay, Cambrex, Walkersville, MD, USA) or the direct quantitation of 3-hydroxymyristic acid by gas chromatography-mass spectrometry (GC-MS) (43). For LAL assay, samples were diluted to fall within the range of the standard curve and heated for 15 min at 70°C to avoid protein inhibition. The quantitation of 3-hydroxymyristic acid was performed as previously described (43), except that 3-hydroxypentadecanoic acid was used as an internal standard in the present studies. GC-MS analysis of 3-hydroxymyristate was performed by using the selected ion monitoring (SIM) mode. The 3-hydroxymyristate concentration was found to correlate significantly with the biological/LAL activity of LPS (44). Areas under the LPS curves (AUC) in total plasma and plasma fractions were calculated from kinetic curves which were measured over 24 hours.

Cytokine and Chemokine Measurements- Cytokines (IL-10; TNFα; interferon-gamma, IFNγ; IL-6) and chemokines (monocyte chemoattractant protein-1, MCP-1) were assayed by cytometric bead array (CBA) using a commercially available kit (CBA mouse inflammation kit, BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instructions.

Leukocyte counts in LPS-injected mice- Total leukocyte count was conducted in a Malassez cell after lysis of red blood cells. An aliquot of blood was used for blood smear prior to May Grünwald Giemsa staining in order to determine the relative proportions of leukocyte subpopulations.

Histological examination- Lung, liver and kidney segments were fixed in 10% v/v phosphate-buffered formalin (pH 7.4) for 24 h and then embedded in paraffin. Five µm-thick sections were stained with hematoxylin and eosin, and viewed by light microscopy at x200 magnification (45).

Ex-vivo response of mouse splenocytes to LPS- Splenocytes were isolated from wild-type or PLTP+/− mice and cultured as previously described (46). Cells from individual mice were cultured in RPMI 1640 medium in the presence (10%) or in the absence of fetal calf serum (Invitrogen, Carlsbad, CA). After 2 hours of incubation, LPS (low dose, 0.1 µg/ml; high dose, 10 µg/ml) was added to culture media. Culture media from LPS-treated splenocytes were collected after 20 hours of incubation and centrifuged at 1500 rpm for 5 min. Supernatants were kept for further cytokine measurements and cytotoxicity assay.

Cytotoxicity- The toxicity of the supernatants from wild-type or PLTP−/− splenocytes treated with the high LPS dose was assessed by determining their impact on the ability of EMT-6J cells to adhere to plastic according to the general procedure previously described (47, 48). Results were expressed as percent of adherent cells, 100% adhesion corresponding to the control value obtained in the absence of LPS.

Lipid and Protein Measurements- Cholesterol and proteins were assayed using commercially available kits (Cholesterol 100, ABX Diagnostics, Montpellier, France, and the Bicinchoninic Acid assay kit, Interchim, Montluçon, France) according to the manufacturer’s instructions. Lipopolysaccharide binding protein (LBP) was assayed by using a commercially available mouse LBP ELISA kit (Cell Sciences, MA, USA).

Statistical Analysis- Results are expressed as mean ± SEM. The statistical significance of differences between two data means was determined with the non-parametric Mann-Whitney or the Wilcoxon tests, as appropriate. The statitical significance of differences between three groups was determined with ANOVA and Fisher test. Survival rates were analysed by the Kaplan-Meier method and compared using the $\chi^2$ test.
RESULTS

PLTP and LBP expression levels in the mouse model. As compared to both WT mouse plasma and human plasma which show high plasma PLTP activity, PLTP\textsuperscript{+/-} mice displayed virtually no plasma phospholipid transfer activity (Fig. 1A and 1B; (40)). PLTP gene knockout was confirmed by PCR analysis with one single, 580-bp product for PLTP\textsuperscript{-/-} homozygotes, and one 580-bp product plus one 680-bp product in PLTP\textsuperscript{+/-} heterozygotes (Figure 1C). Levels of the related LBP were similar in WT and PLTP-deficient plasmas (Fig. 1D).

Tissue abnormalities in LPS-injected mice. In a first attempt to characterize the ability of LPS to cause tissue damage, sections of liver, lung and kidney from injected WT and PLTP\textsuperscript{-/-} mice were stained with hematoxylin/eosin and viewed by light microscopy. As expected from previous studies (45), and 24 hours after intraperitoneal injection of one single dose of \textit{E. Coli} 055:B5 LPS, tissue sections from both WT mice and PLTP\textsuperscript{-/-} mice showed the main characteristics of inflammatory damage (Figure 2). In particular, it is illustrated by sinusoid dilatation and intrasinusoidal thrombi in the liver and by alveolar thickening and the presence of peribronchiolar leukocytes in the lung. Interestingly, accumulation of red blood cells in glomeruli was found in both genotypes, and quantitative analysis revealed a more severe trait in LPS-injected PLTP\textsuperscript{-/-} mice than in LPS-injected WT mice (Figure 2I). Total, polymonuclear, and mononuclear cell counts did not differ significantly between WT and PLTP\textsuperscript{-/-} mice, whether they were treated or not with LPS (Table I). As expected (49), LPS injection produced significant decreases in the number of blood leukocytes. The effect tended to be more pronounced in PLTP\textsuperscript{-/-} mice than in WT mice in which only mononuclear cell counts were decreased, while total white blood, polymonuclear and mononuclear were all significantly decreased after LPS treatment in PLTP\textsuperscript{-/-} mice (Table I).

LPS-mediated production of cytokines/chemokines in WT and PLTP-deficient mice. To further evaluate whether PLTP may modulate LPS toxicity in vivo, the immuno-inflammatory response to LPS was compared in PLTP\textsuperscript{+/-} and WT mice. To this end, WT and PLTP\textsuperscript{+/-} mice were injected intraperitoneally with 5 mg per kg body weight of \textit{E. Coli} 055:B5 LPS and blood was then collected at indicated times. IL-10, TNF\textgreek{a}, IFN\textgreek{g}, IL-6, and MCP-1 in plasma were assayed by cytometric bead array. At baseline (t=0), only trace amounts of IL-10, TNF\textgreek{a}, IFN\textgreek{g}, IL-6 and MCP-1 were found in plasma of both WT and PLTP\textsuperscript{+/-} mice (Fig. 3A-E). As expected (5, 22, 34, 37), the injection of LPS produced a burst of pro-inflammatory cytokines/chemokines in WT mice at t=1.5h, and a similar trend was observed in PLTP\textsuperscript{+/-} mice (Fig. 3A-E). After 9 hours, plasma levels of IFN\textgreek{g} (Fig. 3B), TNF\textgreek{a} (Fig. 3C), IL-6 (Fig. 3D), and MCP-1 (Fig. 3E) in PLTP\textsuperscript{+/-} mice were approximately twice those in WT mice, while the LPS-induced increases in the concentration of the anti-inflammatory IL-10 were of similar magnitude in WT and PLTP\textsuperscript{-/-} mice (Fig. 3A). After 24 hours, plasma levels of IL-6 and MCP-1 in PLTP\textsuperscript{-/-} mice were still significantly higher than those in WT mice (Fig. 3D and 3E).

Synergistic effects of PLTP and lipoprotein acceptors on LPS-mediated inflammation. Splenocytes were isolated from WT and PLTP\textsuperscript{-/-} mice, and they were treated or not with LPS. As shown in figure 4, phospholipid transfer activity was detected in the culture medium of WT splenocytes, whereas no transfer activity was detected in the medium of PLTP\textsuperscript{-/-} splenocytes. In the absence of fetal calf serum (Figure 5A-5C), addition of LPS (high dose - 10 \textmu g/ml) to cultured cells induced the production of proinflammatory cytokines after 20 hours of LPS treatment. Identical patterns were observed whether the cells expressed PLTP or not, indicating similar intrinsic responsiveness of PLTP\textsuperscript{-/-} versus WT cells. In addition, at the high, 10\textmu g/ml dose, LPS induced cytotoxicity as assessed by a major decrease in the adherence of EMT-6J target cells in both genotypes (Figure 5D). When splenocytes were cultured in the presence of fetal calf serum as a source of lipoproteins (Figure 5E-5H), production of proinflammatory cytokines as well as cytotoxicity after addition of the high, 10\textmu g/ml LPS dose were significantly higher in PLTP\textsuperscript{-/-} than in WT mice. When LPS was used at a lower, 0.1\textmu g/ml dose in complementary experiments, again more pronounced,
significant increases in cytokines production were observed in the presence of 10% calf serum than in serum-free medium (Figure 6). Altogether, these observations indicate that cell-derived PLTP has the ability to moderate the local inflammatory response induced by LPS, but mainly when lipoprotein acceptors are present in the incubation medium.

Modification of LPS distribution and clearance in PLTP-deficient mice. To bring insights into the combined effect of PLTP and plasma lipoproteins on LPS metabolism in vivo, mice were injected with LPS (at a single dose of 0.5 or 5 mg/kg) and the LPS concentration in total plasma or ultracentrifugally isolated lipoprotein fractions was determined either by the quantitation of 3-hydroxymyristic acid (Figure 7) or by the LAL method (Figure 8) over a 24h period. In WT, PLTP+/– heterozygotes, and PLTP−/– homozygotes, time course of plasma LPS concentration after one single, 0.5 mg/kg injection of LPS appeared to be dependent on PLTP expression (Figure 7). As compared to WT controls, 3-hydroxymyristate tended to accumulate in plasma of PLTP−/– mice with no detectable PLTP activity and in which the level of HDL cholesterol is approximately a third that of WT mice (40). The values for 3-hydroxymyristate concentration in PLTP+/– heterozygotes (i.e. with a 30% decrease in plasma phospholipid transfer activity but with no change in HDL cholesterol as compared to WT mice (40)) were intermediate between those measured in WT mice and PLTP−/– homozygotes (Figure 7A, 7B). Finally, complete and partial PLTP deficiencies were associated with decreases in the biliary content of 3-hydroxymyristic acid after 24 hours (biliary 3-hydroxymyristic acid: 117.4±37.7 ng/ml in WT, 82.7±15.3 in PLTP+/–, and 42.5±2.4 ng/ml in PLTP−/–; P<0.01 for PLTP−/– versus WT).

By injecting the high, 5 mg/kg dose of LPS to WT and PLTP+/– animals, again a transient increase in total plasma LPS concentration was observed, and over the entire 24-h period studied, a sustained 2.5-fold higher plasma level of LPS in PLTP−/– mice than in WT controls was observed (Fig. 8A, 8B). In both PLTP+/– and WT mice, plasma LPS returned to barely detectable levels 48h and 96h after LPS injection (data not shown). In both genotypes, plasma LPS was mostly associated with HDL and LFF, with less than 0.1% of total plasma LPS associated with VLDL or LDL (data not shown). At t=1.5 h, the LPS content of LFF was 5.5 times higher (Figure 8C) and the LPS content of HDL was 5.5 times lower (Figure 8E) in PLTP−/– than in WT mice, corresponding to a 21-fold decrease in the HDL to LFF lipopolysaccharide ratio (data not shown). Calculation of areas under the curves indicates that the transfer of LPS from LFF to HDL was actually delayed in PLTP−/– mice (Figure 8).

Increased lethal endotoxemia in PLTP-deficient mice. Survival studies were performed in WT and PLTP+/– mice which were treated with 5 mg per kg body weight of E. Coli 055:B5 LPS. Whereas most of the WT mice survived, 50% and 75% of the PLTP−/– mice had died at only three and seven days respectively after the injection of LPS (Figure 9).

DISCUSSION

Excessive cellular response to large amounts of LPS is deleterious for the host through secretion of numerous pro-inflammatory mediators and multiple organ failure, leading to a high rate of mortality (2-4). However, delivery of LPS to circulating lipoproteins leads to neutralization of its toxic properties (17-28). With regard to LPS clearance, earlier studies reported that kinetic might well be dependent on the amount of LPS injected as well as on the nature of LPS (i.e. native LPS in outer membranes/whole bacteria versus extracted LPS) (50). In earlier studies (2), intravenous administration of a very low dose (2 ng/kg) of an endotoxin preparation to human volunteers produced a transient endotoxemia that was detected only 5 to 15 minutes after administration. In these studies, LPS was dissolved in saline and was not associated with lipoprotein carriers, suggesting that non-lipoprotein-associated LPS might be rapidly cleared from the circulation. However, subsequent animal studies demonstrated that lipoproteins can significantly alter the in vivo metabolism of endotoxins when injected at higher doses (from 7 to 2000 microg/kg) in animal models. Both chylomicrons and HDL were found to bind LPS, leading to an acceleration of their clearance from plasma, and to an increase in their uptake by the liver (17-28). Thus, identification of the molecular mechanisms governing LPS trafficking and inactivation is of extreme importance. Here, we demonstrate for the first time in vivo that...
plasma PLTP plays a key role in transferring LPS to lipoproteins, and thus in neutralizing its activity. In the present study, PLTP deficiency in mice was associated with a significant delay in the transfer of LPS from the lipoprotein-free fraction towards HDL, resulting in an impairment of LPS clearance from the plasma compartment and a significant 3-fold reduction in the LPS concentration in the bile of PLTP−/− mice after 24 hours. Concomitantly, PLTP−/− mice displayed an extended increase in the plasma level of pro-inflammatory cytokines, and eventually a marked decrease in their survival rate as compared to WT animals. Overall, these observations are consistent with an early role of PLTP in the transformation of LPS from its toxic, non-lipoprotein associated form into HDL-LPS complexes in vivo. The present study extends earlier reports which demonstrated that LPS is inactivated when it binds to lipoprotein particles (17-23). Since binding occurs more slowly in the PLTP-deficient state, PLTP arises here as a unique candidate in accelerating the lipoprotein-association and neutralization of LPS in vivo, leading to the inhibition of inflammation. In the present studies, the documented hypoalphalipoproteinemic state of PLTP-deficient homozygotes per se (40) is likely to have contributed in part to the observed phenotype, and as predicted from earlier in vitro experiments (32-34), the selective, inhibitory effect of PLTP on the inflammatory and deadly response to LPS could only operate in the presence of lipoprotein acceptors. Interestingly however, a detrimental effect of partial PLTP deficiency on LPS metabolism could be observed in PLTP+/− heterozygous mice in which a 30% reduction in plasma PLTP activity is not accompanied by a significant decrease in HDL levels (40). Complementary ex vivo studies on isolated splenocytes incubated in the presence of a constant amount of serum in the medium (i.e. in the presence of identical amounts of lipoprotein acceptors) still revealed significant PLTP-related changes in the inflammatory and deadly response to LPS, further demonstrating that PLTP plays its proper role in LPS neutralization.

Overall, the present observations are in agreement with the inability of PLTP to transfer LPS to CD14 and to induce the monocyte response in vitro (32, 35). In contrast to PLTP, which does not activate CD14, the related LBP was found in earlier studies to display a paradoxical dual function, either enhancing the detoxification of endotoxins by lipoproteins or sensitizing the immune system in the case of severe endotoxemia (7, 9, 11, 34, 37). In the plasma of WT and LBP-deficient mice injected with LPS, no significant differences in plasma LPS clearance (6) and TNFα production (37) were observed. Moreover, whereas lethal endotoxemia in mice was prevented by the injection of anti-LBP monoclonal antibodies (5), increased mortality as well as the uncontrolled multiplication and spread of the bacteria was observed in LBP knocked-out mice after intraperitoneal injection of bacteria (6). BPI, another PLTP-related protein which neutralizes LPS, is a potent inhibitor of CD14-dependent cell activation by endotoxins, has direct Gram-negative bacterial killing properties, and enhances bacterial phagocytosis (36, 51). Although BPI is normally an intracellular factor which is produced and stored in neutrophils, and is not detected in plasma under normal physiological conditions (52), the overexpression of circulating forms of BPI or BPI fragments in mice was shown to provide protection from lethal endotoxemia (53, 54). In humans, the injection of recombinant BPI was found to be effective in decreasing morbidity and functional outcomes in children with severe meningococcemia, but with no significant decrease in mortality (55). In contrast to BPI, PLTP is a naturally secreted protein that circulates in the blood stream mainly in association with HDL (56). Unlike LBP, PLTP has no CD14 activation potential (35). Compared to LBP and BPI, PLTP appears in the present study to be the most physiologically relevant candidate to prevent endotoxic shock. It is concluded that plasma PLTP is able to detoxify LPS at a very early, and reversible phase of endotoxic shock. In this context, PLTP operates in close cooperation with the scavenging properties of lipoprotein acceptors that have been previously recognized as key vehicles in LPS transport (19, 22, 23, 30, 57). Increasing the circulating level of PLTP arises here as a new and promising strategy in preventing endotoxic shock.
REFERENCES

FOOTNOTES

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All experiments involving animals were performed in accordance with institutional guidelines and approved by the University of Burgundy’s Ethical Committee on the Use of Laboratory Animals (protocol number 2606).

The abbreviations used are: AU, arbitrary units; AUC, area under the curve; BPI, bactericidal permeability increasing protein; CBA, cytometric bead array; CD14, Cluster of differentiation-14; CETP, cholesterol ester transfer protein; EU, endotoxin units; HDL, high density lipoproteins; HuPLTPTg, human phospholipid transfer protein transgenic; IFNγ, interferon-gamma; IL, interleukin; LAL, Limulus Amebocyte Lysate; LBP, lipopolysaccharide-binding protein; LDL, low density lipoproteins; LFF, lipoprotein-free fraction; LPS, lipopolysaccharide; LT/LBP, lipid transfer/lipopolysaccharide binding proteins; MCP-1, monocyte chemoattractant protein; PLTP, phospholipid transfer protein; PLTP−/−, phospholipid transfer protein-knocked out; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor-alpha; VLDL, very low density lipoproteins; WT, wild-type.

FIGURE LEGENDS

Fig. 1. Plasma from PLTP−/− mice displays low phospholipid transfer activity levels but normal LBP levels as compared to plasma from WT mice. As compared to WT mice (n=7) and normolipidemic human plasmas (n=13), PLTP−/− homozygous mice (n=7) displayed barely detectable plasma PLTP activity, measured through the transfer of fluorescent phospholipids from donor to acceptor particles (A and B). The PLTP knock out was confirmed by agarose gel electrophoresis of PCR products with a single, 580bp band in WT mice (C, track 3), one 580bp band plus one 680bp band in PLTP+/− heterozygotes (C, track 2), and one single, 680bp band with PLTP−/− homozygotes (C, track 1). The circulating levels of the related LBP measured by ELISA were remarkably similar in WT and PLTP−/− plasma (D). AU, arbitrary units; Error bars indicate s.e.m. A-D: See Methods for additional experimental details.

Fig. 2. Tissue abnormalities in LPS-injected mice. Liver from LPS-injected mice shows granular degenerescence of hepatocytes (A – WT), increased congestion noted by the presence of red blood cells in hepatic sinusoids (B - WT), and intrasinusoidal thrombi (C – PLTP+/−). Lung from LPS-injected mice shows increased septal cellularity due to inflammation (D - PLTP+/−), congestion (E, WT) and peribronchiolar inflammatory infiltrate (F, WT). Kidney from WT (G) and PLTP+/− (H) LPS-injected mice shows increased congestion noted by the presence of red blood cells in glomeruli. Individual panels are representative sections from 5 animals in each group. Horizontal bar = 100 μm. Bar graph (I) shows a quantitative analysis of glomerular congestion, as figured by the presence of red blood cells, in WT and PLTP+/− LPS-injected mice; * p < 0.05 vs WT mice (n=5).

Fig. 3. PLTP deficiency is associated with extended cytokine/chemokine increases during endotoxic shock in mice. WT mice (n=7) and PLTP+/− mice (n=7) were injected intraperitoneally with a single dose of LPS (5 mg/kg of body weight). Blood was collected at indicated times and plasma levels of pro-inflammatory cytokines/chemokine (A, IL-10; B, IFNγ; C, TNFα; D, IL-6; and E, MCP-1) were measured by cytometric bead array. Nine hours after injection, plasma levels of IFNγ, TNFα, IL-6, and MCP-1 were twice as high in PLTP+/− (n=7) as in WT (n=7) mice (A-E). Error bars indicate s.e.m. See Methods for additional experimental details.
Fig. 4. WT splenocytes, but not PLTP−/− splenocytes express active PLTP. Splenocytes from wild-type and PLTP+/− mice were maintained for 2 hours at 37°C in the absence of LPS. PLTP activity in culture medium was assayed over a 30-min period by using the fluorimetric assay described under ‘Materials and Methods’. Duplicate curves for WT and PLTP−/− are shown.

Fig. 5. Effect of LPS on pro-inflammatory and cytotoxic response of WT and PLTP+/− splenocytes incubated with LPS at a high dose. Splenocytes from wild-type and PLTP+/− mice were incubated (t=20) or not (t=0) in the presence of LPS (high dose, 10μg/ml). Cytokine (IL-6, A and E; TNFα, B and F; IFNγ, C and G) contents of supernatants were measured as described under ‘Materials and Methods’. LPS treatment was performed in the absence (‘serum-free’) or in the presence (‘10% serum’) of fetal calf serum as a source of lipoprotein. Bars are mean±s.e.m of 5 determinations. * P<0.05 vs WT.

Fig. 6. Effect of LPS on pro-inflammatory and cytotoxic response of WT and PLTP+/− splenocytes incubated with LPS at a low dose. Splenocytes from wild-type and PLTP+/− mice were incubated (t=20) or not (t=0) in the presence of LPS (low dose, 0.1μg/ml). Cytokines (IL-6, TNFα, and IFNγ) contents of supernatants were measured as described under ‘Materials and Methods’. LPS treatment was performed in the absence (‘serum-free’) or in the presence (‘10% serum’) of fetal calf serum as a lipoprotein source. Bars are mean±s.e.m of 5 determinations. * P<0.05 vs WT.

Fig. 7. PLTP deficiency impairs the plasma clearance of LPS/3-hydroxymyristate injected at a low dose. WT (n=7), PLTP+/− (n=7), and PLTP−/− (n=7) mice were injected intraperitoneally with a single dose of LPS (0.5 mg/kg of body weight) and blood was collected at indicated times. LPS content of plasma was evaluated by the GC-MS quantitation of 3-hydroxymyristate (see ‘Materials and Methods’). LPS area under the curve (AUC) in total plasma was calculated from kinetic curves measured over 24 hours. Transient increase in total plasma LPS concentration 1.5 hour after intraperitoneal injection was greater in PLTP+/− heterozygotes than in WT mice (A). The AUC of LPS was significantly higher in PLTP+/− homozygotes as compared to WT mice, and it was intermediate in PLTP+/− heterozygotes. Error bars indicate s.e.m. a Significance of the difference between PLTP+/− heterozygotes and WT controls, P=0.056; b Significance of the difference between PLTP+/− homozygotes and WT mice, P<0.01; c Significance of the difference between PLTP+/− homozygotes and WT mice, P<0.01. See Methods for additional experimental details.

Fig. 8. PLTP deficiency impairs plasma clearance of LPS and inhibits its transfer from LFF to HDL in mice. WT (n=7) and PLTP−/− (n=7) mice were injected intraperitoneally with a single dose of LPS (high dose, 5 mg/kg of body weight) and blood was collected at indicated times. LPS content of plasma (A) or ultracentrifugally isolated fractions (LFF: C and HDL: E) was measured by the LAL assay. LPS area under the curve (AUC) in total plasma (B), LFF (D) and HDL (F) fractions were calculated from kinetic curves measured over 24 hours. (A and B), Transient increase in total plasma LPS concentration after intraperitoneal injection was greater in PLTP+/− mice than in WT mice at 1.5, 9 and 24 hours. (C and D), LPS removal from LFF was slower in PLTP−/− mice than in WT mice. (E and F), PLTP deficiency was associated with fewer molecules of LPS in HDL at the early time: 1.5 hours. Decrease in HDL-LPS between 9 and 24 hours was identical in WT and PLTP−/− mice. Error bars indicate s.e.m. A-F: See Methods for additional experimental details.

Fig. 9. Plasma PLTP deficiency decreases survival to endotoxic shock in mice. As compared to WT mice (n=15), PLTP−/− mice (n=16) were more susceptible to death from endotoxic shock during the 8 day-period after a single injection of LPS (5 mg/kg of body weight). Survival rates were analysed by the Kaplan-Meier method and compared using the χ² test. A and B: See Methods for additional experimental details.
Table 1. Leukocyte counts in LPS-injected mice.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>PLTP /-</th>
</tr>
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<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total white blood cells</td>
<td>4920 ± 553</td>
<td>6110 ± 1183</td>
</tr>
<tr>
<td>Polynuclear cells</td>
<td>3140 ± 383</td>
<td>2501 ± 397</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>1780 ± 182</td>
<td>3609 ± 1048</td>
</tr>
<tr>
<td><strong>24 hours</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total white blood cells</td>
<td>1730 ± 460</td>
<td>1400 ± 272&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Polynuclear cells</td>
<td>1288 ± 310</td>
<td>1025 ± 250&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>440 ± 159&lt;sup&gt;a&lt;/sup&gt;</td>
<td>384 ± 40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Wild-type (n = 5) and PLTP /- mice (n = 5) were injected intraperitoneally with LPS (5mg/kg). Blood samples were drawn before (baseline) and 24 hours after LPS injection. Leukocyte count was performed as described in Experimental Procedures. Results are expressed as cells per mm<sup>3</sup> and are means ± SEM. <sup>a</sup> Significance of the difference from baseline counterparts: P<0.05.
Figure 1. Gautier et al.
Figure 2. Gautier et al.
Figure 3. Gautier et al.
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Figure 5. Gautier et al.
Figure 6. Gautier et al.
Figure 7. Gautier et al.
Figure 8. Gautier et al.
Figure 9. Gautier et al.
Effect of plasma phospholipid transfer protein deficiency on lethal endotoxemia in mice

Thomas Gautier, Alexis Klein, Valérie Deckert, Catherine Desrumaux, Nicolas Ogier, Anne-Laure Sberna, Catherine Paul, Naig Le Guern, Anne Athias, Thomas Montange, Serge Monier, Françoise Piard, Xian-Cheng Jiang, David Masson and Laurent Lagrost

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