Host defences against metabolic endotoxaemia and their impact on lipopolysaccharide detection

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Abstract

Bacterial endotoxin (lipopolysaccharide, LPS), is one of the most potent inducers of inflammatory signalling, yet it is abundant in the human gut and the modern diet. Small quantities of LPS routinely translocate from the gut lumen to the circulation (so-called 'metabolic endotoxaemia'), and elevated plasma LPS concentrations are reported in a variety of chronic non-communicable diseases, including obesity, non-alcoholic fatty liver disease, atherosclerosis and type II diabetes. Murine models of experimentally-induced endotoxaemia and Toll-like receptor-4 deficiency suggest that endotoxin may promote the metabolic disturbances that underpin these diseases. However, as bioactive LPS is cleared rapidly from the circulation, and reported levels of endotoxin in human plasma vary widely, the potential relevance of metabolic endotoxaemia to human disease remains unclear. We here review insight into these questions gained from human and murine models of experimental endotoxaemia, focussing on the kinetics of LPS neutralisation and its clearance from blood, the limitations of the widely used limulus assay and alternative methods for LPS quantitation. We conclude that although new methods for LPS measurement will be required to definitively quantify the extent of metabolic endotoxaemia in man, evidence from numerous approaches suggests that this molecule may play a key role in the development of diverse metabolic diseases.

1) Background

Bacterial endotoxins (lipopolysaccharides, LPS), are structurally conserved amphiphiles which are essential for the functional integrity of the outer membrane of Gram-negative bacteria [1]. As such, endotoxins represent key targets for the innate immune system, triggering inflammatory signalling at picomolar concentrations in man [2,3]. Although the human large intestine contains large quantities of LPS derived from the commensal microbiota, blood levels of the molecule are thought to be very low in health [4,5]. However, circulating endotoxin concentrations can increase dramatically during Gram-negative sepsis [6], and much research interest has focussed on the role of endotoxaemia in provoking a condition referred to as the systemic inflammatory response syndrome (SIRS), which is characterised by excessive production of pro-inflammatory cytokines and potentially life-threatening impairments of organ and vascular function [7].

More recently, interest has grown in the potential of much lower doses of endotoxin to potentiate the sub-clinical inflammation that is thought to underpin a number of common metabolic disorders. This focus has arisen largely from the results of epidemiological studies, in which plasma or serum LPS levels were quantified in healthy volunteers and patient groups using the limulus amoebocyte lysate (LAL) assay. Numerous studies have reported that even modestly raised levels of circulating endotoxin are associated with incidence or prevalence of atherosclerosis [8-10], type II diabetes [11-13], obesity [14] and non-alcoholic fatty liver disease [15-17]. Moreover, the hypothesis that endotoxin is a causal mediator of these diseases is supported by the observation that experimentally induced low-grade endotoxemia accelerates atherosclerosis in rodents [18,19], and triggers insulin resistance in both mice and human volunteers [20,21]. Accordingly, the findings that mice deficient in LPS-sensing are resistant to diet-induced atherosclerosis [22] and insulin resistance [23,24],

lend further support to the hypothesis. The main source of circulating endotoxin in the absence of infection is debated, but it is most likely to translocate from the gut lumen, where it may be derived from the commensal microbiota [20], or dietary sources [25]. This so-called 'metabolic endotoxemia' is thought to be facillitated by the absorption of dietary fat [26], as supported by recent reports that circulating endotoxin levels may be increased in the post-prandial phase, in both rodents and man [20,27,28].

However, these findings do not sit well with a large body of literature showing that bioactive LPS is cleared rapidly from the circulation [29,30], and that blood levels of LPS in healthy subjects reported in many epidemiological studies are often several orders of magnitude higher than those demonstrated to induce severe inflammation in experimentally-induced endotoxaemia [31]. This review therefore aims to explore potential explanations for the disconnect between these two areas of the literature. The limitations of the LAL assay, potential refinements and the relative merits of alternative means of LPS quantitation, will be discussed. Recent findings made using the LAL assay will be compared with the results of studies using serological, isotope-labelling and mass-spectrometry techniques to establish the mechanisms and kinetics of LPS neutralisation and its clearance from blood. Finally, current understanding of defensive mechanisms against enteric LPS-induced inflammation, and what insight this lends to the study and clinical consequences of metabolic endotoxaemia, will be discussed.

2) Structure and function of lipopolysaccharides

Lipopolysaccharides share a common architecture which generally comprises three major structural domains: a well conserved lipid-A domain, a moderately conserved core oligosaccharide domain and, in most organisms, a highly variable O-polysaccaride domain (Figure 1A).

The lipophilic lipid A domain typically comprises a dimer of D-glucosamine residues, which are phosphorylated at positions 1 and/or 4', and acylated by four (R)-3-hydroxy fatty acids in ester (positions 3 and 3') and amide linkage (positions 2 and 2'). Frequently, two of the glucosamine-linked acyl chains are themselves acylated at the 3-hydroxyl group by secondary fatty acids [32]. This di-glucosamine, hexa-acyl, bis-phosphoryl structure represents the canonical enterobacterial lipid A, which is both the most studied and the most potent of naturally occurring lipid A structures with respect to capacity to stimulate inflammatory signalling in human cells (Figure 1B) [33].

The hydrophilic core and O-polysaccharide domains are exposed to solvent, and are the primary targets for antibodies raised against Gram-negative bacteria [34]. Notably, the antigenic diversity of O-polysaccharides is vast, and a single Gram-negative species may comprise hundreds of strains, each expressing antigenically distinct O-polysaccharides. Rare mutants expressing LPS that lacks O-polysaccharide are referred to as 'rough', to differentiate them from 'smooth' strains which express O-polysaccharide.

Innate immune detection of extracellular LPS is mediated by the recognition of lipid A by two key pattern recognition receptors (PRRs) - myeloid differentiation (MD)-2 and Toll-like receptor (TLR)-4. Through binding to a specialised hydrophobic pocket in MD2, agonist-type lipid A induces a conformational change which is transmitted across the cell membrane by TLR4. This in turn facilitates the induction of NF-κB and interferon regulatory factor (IRF)-3-dependent signalling, leading to rapid upregulation of diverse genes with pro-inflammatory and anti-viral function [35]. A number of specialised lipid shuttle proteins, including LPS-binding protein (LBP) and soluble CD14 (sCD14), enhance delivery of LPS to the TLR4/MD2 complex, so greatly amplifying cellular sensitivity to low concentrations of LPS [35].

Although the lipid A moiety is the most conserved component of the LPS molecule, variations in this structure do exist between species. In particular, while the hexa-acyl, bis-phosphoryl lipid A structure is the most widely studied variant (because it is expressed by *Escherichia coli*), a number of genera express lipid A molecules with structural features that limit recognition by human TLR4/MD2. Chemical synthesis of lipid A analogues has revealed that the loss of either glucosamine-attached phosphate group from the canonical form results in a ~100-fold reduction in biological activity with respect to cytokine synthesis by human monocytes [33]. Likewise, deletion of one acyl chain, yielding a penta-acyl form, also reduces biological activity by a factor of ~100. Removal of both secondary fatty acids results in a molecule (termed compound 406) which not only lacks agonist activity, but is a potent antagonist of human TLR4/MD2 [33].

Structural studies have explained this loss of activity by showing that when agonist-type lipid A binds MD2, five of the lipid chains are fully accommodated within the hydrophobic pocket, and the sixth (R2) chain is exposed on the surface, so providing a hydrophobic interface for interaction with TLR4, and both lipid A phosphates further stabilise this complex [36]. By contrast, while the tetra-acyl LPS inhibitors Eritoran and compound 406 bind well to the MD2 pocket, no acyl chains are exposed for interaction with TLR4, and they do not cause the conformational change required to trigger TLR4 signalling [37-39].

These observations offer key insight to the study of metabolic endotoxaemia, since the majority of the Gram-negative commensals in the human intestine are of the family Bacteroidaceae, which outnumbers Enterobacteriacea at least 1,000:1 [40]. Notably, members of this group, such as *Bacteroides fragilis*, tend to express an unusual lipid A characterised by a single glucosamine-linked phosphate group and only five acyl chains, several of which demonstrate unusual length and branching (Figure 1C) [41,42]. Accordingly, *B. fragilis* LPS does not stimulate human TLR4/MD2 [43] and inhibits the biological activity of canonical

enterobacterial LPS in human systems [44,45]. Thus, it is likely that the majority (>99%) of LPS molecules derived from the intestinal microbiota are non-stimulatory in man.

3) Experimental models of endotoxaemia

As mentioned above, the main driver for the development of experimental models of endotoxaemia came from the need to improve understanding of the role of LPS in SIRS and Gram-negative sepsis. Such studies have revealed that when blood levels of LPS exceed a threshold concentration (discussed below), myeloid cells - including both circulating monocytes and tissue resident macrophages - are activated systemically to produce excessive quantities of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α [7]. These mediators are thought to play key roles in the negative consequences of SIRS, including fever, leukocytosis, tachycardia, shock, multiple organ failure, and the compensatory anti-inflammatory response syndrome (CARS) which frequently follows [7].

Such responses are readily modelled in human volunteers, through the bolus intravenous injection of *E. coli* endotoxin. Doses between 2 and 4 ng/kg reproducibly result in dramatically increased plasma levels of IL-6 and TNF-α (>500-fold over baseline), leukocytosis, and a range of SIRS symptoms, including fever, chills, myalgia, hypotension and nausea, which resolve within several hours [29,46]. A key lesson learned from these models is that human volunteers are far more responsive to intravenously administered LPS than mice, which require doses per kilogram 250 - 500-fold higher to elicit similar cytokine and physiological responses [47].

More recently, the impact of lower, sub-clinical doses of LPS on metabolic function in man have received more attention. For example, doses of 0.1 to 0.2 ng/kg, which do not induce any outward symptoms of malaise, nevertheless result in modest increases in

circulating pro-inflammatory cytokines (4 to 20-fold increase in IL-6 and TNF-α), leukocyte count and APR markers [3,48]. Such studies have revealed that the human body is responsive to vanishingly small quantities of circulating LPS - significant increases in leukocyte counts, pro-inflammatory cytokines and APR markers are induced by as little as ~2.5 pg/ml LPS (the plasma level achieved by the 0.1 ng/kg dose). Notably, these *in vivo* observations tally well with *in vitro* studies of cultured human whole blood and monocytes, which secrete IL-6 and TNF-α in response to as little as 10 pg/ml enterobacterial LPS [27].

Both human and murine models of experimentally induced endotoxaemia also support a potential role for LPS in metabolic disease. In man, for example, a 35% decrease in insulin sensitivity index was reported 24 h after intravenous injection of 3 ng/kg LPS [21]. In mice, chronic infusion of LPS (300 μg/kg/day) for 4 weeks by means of a subcutaneous osmotic pump also caused significant increases in fasting glucose and insulin levels, hepatic triglyceride content and adiposity [20]. In rats, chronic LPS infusion increased caloric intake and weight gain in a dose-dependent manner [49], and a single dose (100 μg/kg ip) induced leptin resistance [50]. Triglyceride-rich lipoproteins are markedly elevated in rodents after LPS challenge [51], and the capacity of serum to accept cholesterol effluxed from macrophages (a key measure of the reverse cholesterol transport pathway), is significantly impaired in both mice and human volunteers after acute LPS treatment [52]. Accordingly, repeated LPS injections accelerate plaque formation in murine models of atherosclerosis [18,19]. The notion that LPS may potentiate metabolic disease directly is further supported by numerous studies demonstrating that mice deficient in LPS-signalling (via MyD88 or TLR4 deficiency), are resistant to diet-induced adiposity [53,54], insulin resistance [23,55,56], hepatic steatosis [55,57] and atherosclerosis [22,58].

4) Reported levels of circulating LPS in health and disease

These findings have inspired a number of epidemiological studies to seek potential evidence of a role for sub-clinical endotoxaemia in metabolic disease. To date, such studies have addressed this question almost exclusively through use of the LAL assay to quantitate LPS in plasma or serum of healthy volunteers and patient cohorts. Although the LAL assay faces a number of specific challenges in this context (which will be discussed later), numerous reports of associations between serum or plasma LPS measured in this way with incidence or prevalence of metabolic diseases have been made.

For example, subjects in the Bruneck study (n=516) with plasma endotoxin levels at baseline in the 90th percentile (>50 pg/ml), were reported to face a threefold risk of incident carotid atherosclerosis over a five year followup, independent of traditional vascular risk factors [8]. Similar findings were reported by the FINRISK study, which monitored incident cardiovascular disease over a 10 year followup period (n=505). In this study, subjects with plasma endotoxin levels in the highest quartile faced a hazard ratio of 1.82 (95% CI 1.22 to 2.73) versus quartiles 1 to 3, although this association was lost after adjustment for serum lipids [9]. In a study of 30 peritoneal dialysis patients, plasma LPS levels correlated with carotid intima-media thickness (a measure of subclinical atherosclerosis), and were significantly higher in those with established cardiovascular disease than those without [10].

Plasma LPS levels are also reported to be associated with presence or risk of developing type II diabetes mellitus (T2DM). For example, Creely *et al* reported that circulating LPS levels were 76% higher in T2DM patients compared with matched controls (n=25 per group) [11], and Al-Attas *et al* reported that endotoxin levels were significantly higher in patients with T2DM (n=413) than in non-diabetic controls (n=67) [12]. Serum endotoxin levels were also reported to be significantly higher in subjects with prevalent (n = 537) or incident diabetes (n = 462) than nondiabetic individuals, among participants of the FINRISK97 cohort (7,169 subjects followed up over 10 years) [13]. Likewise, serum endotoxin levels were

reportedly higher in patients with non-alcoholic fatty liver disease (NAFLD) [15-17], and in obese subjects [14] than in healthy controls.

These findings have re-inforced the notion that chronically elevated circulating LPS levels may contribute to the risk of developing metabolic disease. However, a fundamental difficulty with the interpretation of these results is that reported concentrations of endotoxin are often dramatically higher in these studies than those demonstrated to trigger SIRS in human experimental endotoxaemia.

A key point to consider when making such comparisons, is that LPS concentrations measured using the LAL assay are reported either as mass per unit volume (e.g. pg/ml), or as Endotoxin Units per unit volume (EU/ml, a measure of bioactivity relative to *E. coli* LPS). Mass-based reporting is prefered by some groups since the findings are immediately comparable with the large body of literature on murine LPS challenge and *in vitro* LPS signalling. Others prefer to report in EU/ml, since the bioactivity of a specific mass of LPS may vary between batches, or when extracted by different methods, and especially when isolated from different types of bacteria [59]. However, because almost all epidemiological studies of plasma LPS and experimentally-induced endotoxaemia have employed *E. coli* LPS for challenge, and as the standard for LAL assay quantitation, a widely used rule of thumb is that 1 EU is equivalent in bioactivity to ~100 pg of *E. coli* smooth LPS. This approximation is widely accepted as valid because all serotypes of *E. coli* LPS express identical, canonical lipid A and show very similar bioactivities in the LAL assay.

A comprehensive overview of the relative merits of those studies which have measured circulating LPS levels in healthy human volunteers is beyond the scope of this review. Those seeking an in-depth exploration of this topic are directed to the excellent recent review by Gnauck and colleagues [31]. Their comprehensive overview identified 44 studies, published between 1984 and 2014, in which LPS was detectable and quantified in plasma or serum of

healthy human volunteers using the LAL assay. Of those studies reporting endotoxin by mass, circulating LPS levels ranged between 0.5 and 65 pg/ml (median 5.1 pg/ml), and concentrations reported in endotoxin units ranged between 0.15 and 61 EU/ml (median 0.32 EU/ml) [31].

Thus, the median reported concentration of endotoxin in blood of healthy human volunteers using the limulus assay is between 2 and 12-fold higher than the dose required to increase plasma pro-inflammatory cytokines and leukocyte count far beyond those measured in healthy subjects (i.e. 2.5 pg/ml or ~0.025 EU/ml, see above). Notably, the magnitude of the increases in circulating cytokines, leukocyte counts and acute phase markers generated by such doses are also far greater than reported in subjects with common metabolic disorders. Thus, a fundamental disconnect exists between reported levels of circulating LPS in most studies of metabolic endotoxaemia, and the concentration required to trigger systemic inflammation determined empirically.

5) Kinetics of LPS bioactivity in the circulation

Insight into potential mechanisms underpinning this discordance may be gained from studies of the kinetics of LPS bioactivity in the circulation. Studies which have used the limulus assay to monitor LPS clearance have shown that endotoxin bioactivity is rapidly cleared following intravenous injection. For example, less than 1% of LAL-reactive LPS remained in the circulation of dogs 5 minutes after injection with LPS [60]. Rats cleared on average 72% of initial bioactivity within 5 minutes post-injection [61]. In baboons receiving 0.1 mg/kg LPS bolus i.v. injection, plasma LAL bioactivity peaked at 5 minutes, but fell by ~75% over the following 5 minutes [62]. Likewise plasma LPS levels were just 13 pg/ml in human volunteers 5 minutes after bolus i.v. injection with 2 ng/kg LPS (which should have yielded an initial plasma concentration of ~56 pg/ml, assuming 2.5 litres plasma volume), and

LPS bioactivity was undetectable at 30 minutes (as shown in Figure 2A) [29]. These experiments suggest that LPS is cleared rapidly from the circulation, with a half-life of several minutes or less in many species, including man. However, alternative approaches to the investigation of the pharmacokinetics of LPS have revealed a more complex scenario.

In particular, many studies using radiolabelled LPS have demonstrated that such preparations exhibit biphasic clearance from the circulation, characterised by an early, rapid clearance similar in kinetics to that observed for LAL bioactivity, followed by a phase of much slower clearance [30,63-66]. For example, the half-life of radiolabelled smooth LPS in the circulation of mice is typically in the range of several minutes during the rapid phase, but this increases to ~10-15 hours during the second phase of clearance [30,65]. Similar half-lives for both phases of clearance has been reported in rats and rabbits [63,64,66]. Notably, evidence suggests that rough LPS is cleared even more rapidly from the circulation than smooth LPS, during the alpha phase (as shown in Figure 2B) [30].

Mass-spectrometry, when combined with high-performance liquid chromatography, (LC-MS) can also be used to follow the pharmacokinetics of intravenously administered synthetic lipid A analogues, such as Eritoran (E5564) and its precursor compounds, which were developed as inhibitors of TLR4/MD2 for the treatment of sepsis. Clinical studies have shown that such compounds are cleared relatively slowly from the circulation [67-69]. For example, the elimination half-life of E5564 was 35-55 hours [67,69], and that of ONO-4007, a synthetic lipid A analogue trialled as an antitumor agent, ranged between 74 and 95 hours after intravenous injection [70]. However, mirroring the observations of the rapid loss of LAL reactivity following injection of LPS despite a continuing presence of intact LPS molecules, these studies also show that the capacity of E5564 to inhibit LPS signalling is lost within minutes of addition to blood *ex vivo* [67,71]. Moreover, LC-MS shows conclusively that this is not due to chemical degradation of lipid A analogues, which is negligible in the

circulation [67,71], adding further weight to evidence from SDS-PAGE experiments showing no major cleavage of the glycolipid structure of radiolabelled LPS in the circulation up to 3 hours post injection [64], and the recovery of LPS biological activity after re-extraction from plasma [72].

Together, these findings suggest that the bioactivity of LPS (as measured by the LAL assay) and lipid A analogues (as measured by capacity to inhibit TLR4/MD2) is diminished rapidly in blood. However, this is not due to chemical degradation and is only partly explained by their clearance from the circulation. Thus, much of the reduction in bioactivity of LPS and lipid A analogues in the circulation arises from their reversible inactivation by plasma components.

6) Neutralisation of LPS bioactivity by plasma components

Early insight into this paradoxical loss of activity without structural alteration came from experiments employing density gradient centrifugation of plasma. These showed that LPS added to blood *in vitro* or *in vivo* partitions quickly into circulating lipoproteins, such as low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), high-density lipoprotein (HDL) and chylomicrons (CM) [71,73]. This process, which does not lead to endotoxin degradation, occurs rapidly. For example, the majority of ^{125}I-labelled LPS infused into rabbits partitioned into a low density fraction (ρ<1.2 g/cm^3) in less than one minute, and by 30 minutes essentially all remaining circulating LPS was found in this fraction [64]. Similar kinetics were reported for human blood [74]. In rodents, the majority of LPS added to blood partitions to HDL [66,75], while in man, a considerable proportion is also shuttled to LDL, VLDL and chylomicrons [71,74,76,77].

Structural studies have shown that this association is mediated by the intercalation of the hydrophobic lipid A domain into the outer phospholipid leaflet of lipoprotein particles [73].

As such, the domain of the LPS molecule that is detected by the enzymes of the limulus assay, and the human innate immune system, is sequestered from detection. Indeed, the capacity of plasma to neutralise LPS activity in the LAL assay is readily reproduced by incubation with isolated lipoproteins - including LDL, VLDL, HDL and CM [78-80].

The capacity of LPS to trigger inflammatory signalling is also blunted by adsorption to lipoproteins. *In vitro* studies have shown that pre-incubation of LPS with serum or lipoproteins significantly reduces its capacity to stimulate cytokine release by monocytes and macrophages [81,82]. For example, the capacity of LPS to stimulate IL-1β, IL-6 and TNF-α production by human monocytes and peritoneal macrophages was 20- to 1,000-fold lower following pre-incubation with rabbit serum or the lipoprotein fraction [82]. Likewise, the threshold concentration of LPS required to stimulate IL-1 β release from human monocytes was increased by 10 to 100-fold following pre-incubation in human serum [81]. Notably, human serum was able to inhibit IL-1β release in response to many different types of LPS and free lipid-A, but required several hours of pre-incubation to yield maximal inhibition [81]. The kinetics of lipoprotein complexing are therefore much slower than the time required for LPS to induce activation and cytokine production by human monocytes (seconds) [27,83].

Nevertheless, *in vivo* models also support the potential of lipoproteins to reduce the inflammatory impact of circulating endotoxin. For example, pre-incubation of LPS with VLDL, LDL, HDL or chylomicrons was shown to protect against endotoxin-induced death in mice, which is established to be dependent on monokine release (IL-1β, IL-6, TNF-α) by myeloid cells [78]. In keeping with the *in vitro* findings, protection was dependent on duration of pre-incubation with lipoproteins [78]. Likewise, transgenic mice engineered to express 2-fold elevated HDL levels, and mice deficient in the LDL-receptor which have high levels of LDL, are protected from LPS-induced death [84,85]. Similar findings were reported for preincubation of LPS with chylomicrons in rats [86,87]. In man, the preincubation of LPS

with autologous blood significantly reduced subsequent fever, leukocyte and cytokine responses to endotoxaemia compared to intravenous administration of free LPS [88]. Likewise, infusion of human volunteers with reconstituted HDL particles significantly reduced the production of cytokines and symptoms of malaise after endotoxin challenge [89].

Although these studies clearly point to a primary role for lipoprotein particles in the neutralisation of LPS by serum, it should be noted that LPS also binds to many other plasma proteins, including albumin, complement, soluble CD14, transferrin, LPS-binding protein (LBP), phospholipid transfer protein (PLTP), bactericidal permeability-increasing protein, apoA-I, apoE, apoA-IV and (O-antigen specific) immunoglobulin [90]. Although many of these proteins have been shown to inhibit recognition of LPS by the LAL assay [91,92], they do not appear to play a major role in the capacity of serum to blunt inflammatory cytokine production by LPS [81,93]. This point is made evident by the observation that incubation of LPS with lipoprotein-deficient serum for several hours did not blunt the capacity of LPS to stimulate monocyte IL-1β production [81]. LPS neutralisation also could not be replicated by naïve gamma globulin, fibrinogen or albumin [94]. Furthermore, although complement is well established to interact with LPS, it is not likely to play a major role in LPS neutralisation by serum, since procedures which deplete complement from serum, such as heat treatment (56^{o}C for 1 hour), or pre-treatment with yeast, do not diminish the capacity of serum to inhibit the monocyte activating potential of LPS [81]. Nevertheless, the capacity of serum to inhibit the LAL assay (discussed later), varies widely between human volunteers in a manner that is not fully explained by lipoprotein concentrations, and is depleted on exposure to endotoxin while lipoprotein concentrations are not diminished to the same extent [92].

Many previous reports have argued that antibody is not required for, and likely not relevant to, the neutralisation of LPS bioactivity by serum [81,93,94]. However, it should be noted that most of these studies have examined serum from animals which had no prior

exposure to the type of LPS used experimentally (i.e. naïve serum was used). It is well established that two distinct phases of tolerance to LPS arise in animals following treatment. The early phase, which lasts ~1-2 days and diminishes thereafter, is mediated by cellular refractiveness to TLR-stimulation [95]. The later phase is mediated by antibodies targeting the O-antigen of LPS, which arise from about day 6 after the primary challenge, mainly through T-cell-independent activation of marginal zone B-cells, and involving both class-switching and memory B-cell formation [96-98]. Crucially, while the early phase of tolerance reduces host sensitivity to all forms of LPS, the later phase is highly specific to LPS containing the same O-antigen, as shown in both rabbits and human volunteers [96]. The binding of antibody to LPS reduces macrophage cytokine release by such complexes, in a manner thought to involve redirection of LPS from pathways leading to inflammatory cytokine production, towards degradative, non-inflammatory pathways [99,100]. Accordingly, LPS-specific antibodies have been shown to protect mice from lethal endotoxaemia [101]. Further evidence that specific antibodies may contribute to the neutralisation of LPS bioactivity is found in the observation of very strong correlations ($P<0.001$) between titres of IgG and IgM antibodies targeting the core saccharide epitopes of *E. coli* LPS, and the capacity of human serum to inhibit LAL assay responses to *E. coli* LPS [102].

These findings are potentially of much relevance to the concept of metabolic endotoxaemia, since both mice and humans express IgA targeting commensal antigens (including LPS); thus likely limiting the translocation of commensal LPS from the gut [103,104]. In terms of clearance of LPS from the circulation, it is notable that while mice generally lack systemic IgG antibody directed against commensal antigens [105], human plasma contains IgG targeting commensal antigens, including LPS [81,102,106]. Thus, O-

polysaccharide-specific antibody titre is likely to play a key role in the clearance and physiological consequences of metabolic endotoxaemia.

7) Tissue distribution of LPS cleared from the circulation

Most studies examining the *in vivo* fate of intravenously administered radiolabelled LPS have reported that by far the majority of radiolabel cleared from plasma is found in the liver after injection [64,107-110]. Much smaller quantities of LPS are taken up by lung, spleen, ovary, adrenal gland and skeletal muscle [64,75,109]. Of the fraction of radiolabel that remains in blood after the primary clearance phase, very little is found in leukocytes, with the majority (>95%) in platelet poor plasma, complexed to lipoproteins as described above [63,64].

Numerous experimental approaches have shown that Kupffer cells (hepatic macrophages) are the principal cell type responsible for the removal of smooth-type LPS during the primary clearance phase. For example, autoradiography has shown that Kupffer cells take up the majority of radiolabelled LPS following intravenous injection in rodents, with very little uptake observed in endothelial cellls or hepatocytes [64,111,112]. Immunostaining using the LPS-binding protein Factor C confirmed the rapid localisation of injected LPS within Kupffer cells [61], and depletion of Kupffer cells using clodronate liposomes markedly reduced hepatic uptake of LPS [109].

However, evidence suggests that more lipophilic forms of LPS (rough LPS and lipid A) may be taken up to some degree by hepatocytes [113,114]. The slower clearing lipoprotein-bound LPS is also thought to be taken up primarily by hepatocytes [115]. Indeed, experimentally formed complexes of LPS and chylomicrons are thought to bypass Kupffer cells (and consequently inflammatory cytokine production), shunting a greater proportion through hepatocytes for excretion in bile [86,116]. Likewise, complexing LPS to the ApoE

apoprotein before injection redirects LPS uptake from Kupffer cells to liver parenchymal cells [66]. A small proportion of lipoprotein-bound LPS may also be taken up by non-hepatic cells via classical lipoprotein receptors [75]. For example, endocrine tissues, such as ovary and adrenal gland, which absorb HDL to facilitate steroid production, also tend to accumulate a small, but significant, fraction of circulating LPS-HDL complexes [75].

The rapid removal of free LPS during the primary clearance phase is thought to be mediated primarily by scavenger receptors which are highly expressed by Kupffer cells. For example, the co-administration of scavenger-receptor ligands acetylated LDL, polyinosinic acid and maleylated BSA each inhibited hepatic uptake of lipid IVA by up to 50% in mice [107]. LPS uptake by Kupffer cells cultured *in vitro* was also inhibited to a similar degree by a range of different polyanionic scavenger receptor ligands [117]. Immunoglobulin Fc receptors are also likely to be involved in the primary phase of LPS clearance, since mice deficient in antibody production (RAG-$2^{-/-}$ and $Btk^{-/-}$) show impaired LPS clearance which is restored by reconstitution with normal mouse IgM [118]. LPS uptake by cultured macrophages is also greatly increased by complexing with specific antibody [119]. Complement receptors, however, does not seem to be key players, since hepatic uptake of LPS is unaltered by complement depletion with cobra venom factor [114].

The removal of lipoprotein-bound LPS from the circulation appears to be mediated mainly by the established lipoprotein clearance receptors (e.g. LDL-receptor, SR-BI etc) [66,86,115,116]. This is consistent with the close similarity in kinetics between second phase LPS clearance and normal lipoprotein turnover in mice and humans. Indeed, the rapid clearance phase is absent when preformed LPS-lipoprotein complexes are injected into mice [64], and mice lacking the HDL receptor SR-BI show delayed LPS clearance [120]. Compared to free LPS, lipoprotein-bound LPS is also poorly absorbed by macrophages *in vitro* [82].

The fate of LPS following delivery to hepatocytes is excretion in bile, where it begins to accumulate within one hour of intravenous administration [112]. This pathway accounts for the majority of LPS eliminated from the body [64,109,110,112], and only a small proportion of lipid-A derived ^{3}H-labelled fatty acids are recovered in urine [109,121]. Although bile acids mask the detection of LPS by the LAL assay, re-extraction methods reveal that LPS in bile retains bioactivity with respect to lethality in galactosamine-sensitised mice [112]. This suggests that much of the LPS delivered to bile arrives with limited structural degradation. LPS that has been taken up by Kupffer cells is also eventually transferred to hepatocytes [72,113]. Although the mechanism of this transfer is not clear, it has been observed that macrophages cultured *in vitro* rapidly "efflux" LPS from their cell surface to lipoprotein particles [119,122,123], in a manner that seems to be dependent on the transporter ABCA-1 [124]. Thus, the clearance of LPS from macrophages follows a path that is strikingly similar to that used to remove excess cellular cholesterol, i.e. "efflux" via an ABC transporter to lipoproteins, especially HDL, redistribution among other classes of lipoprotein via lipid transferases, uptake of the complex via lipoprotein receptors on hepatocytes and, eventually, excretion to bile.

8) Enzymatic neutralisation of LPS

As discussed above, the re-extraction of LPS complexed to plasma lipoproteins reveals little evidence of structural degradation over several hours [64,72]. However, LPS can be degraded following uptake by macrophages or the liver. Much has been learned of this process through the use of radiolabelled LPS - most commonly by labelling the fatty acids with $^{3-}$H, the glucosamine backbone with $^{14-}$C and the phosphate groups with $^{32-}$P. Such studies have shown that while the di-glucosamine and core saccharides remain quite stable as

LPS is processed in the liver or in macrophages cultured *in vitro*, the lipid-A attached phosphates and secondary fatty acids are relatively prone to cleavage [109,125].

For example, SDS-PAGE of LPS extracted from liver revealed no major cleavage of the basic glycolipid structure within 3 hours [64], and the O-polysaccharide was found to remain antigenically intact in the liver for several days after LPS treatment [72,113]. Although some workers have reported partial degradation of O-polysaccharide over days to weeks in liver or cultured macrophages [72,126,127], others have reported that it can remain antigenically intact in monocytes or macrophages cultured *in vitro* for weeks to months after challenge [127,128].

The secondary fatty acids, on the other hand, are released more readily. This is achieved largely by the enzyme acyloxyacyl hydrolase (AOAH), which selectively removes these moieties from LPS or lipid A, while leaving the glucosamine-linked fatty acids intact [109,129,130]. Notably, the structure remaining after AOAH treatment of canonical lipid-A is chemically identical to lipid-IVa, the tetra-acyl bis-phosphorylated structure that is a potent inhibitor of human TLR4/MD2 (see Figure 1) [131]. Such deacylated LPS (dLPS) has been shown to inhibit inflammatory cytokine production in response to structurally intact LPS via competition for the upstream LPS shuttling proteins CD14 and LBP [132,133]. Thus, AOAH not only deactivates LPS but yields a product that inhibits the activity of agonist LPS [132]. It has been argued that this pathway may be of little functional relevance in mice, since tetra-acyl LPS remains a weak agonist for murine TLR4/MD2 [134], but global deficiency in AOAH results in dramatic hepatic inflammation and hepatosplenomegaly several days after LPS challenge [109,135]. Kupffer cells are the principal cell-type responsible for deacylation of LPS in the liver, since hepatocytes do not express AOAH activity, and clodronate liposome treatment reduced hepatic deacylation of LPS by greater than 90% [109]. Secondary fatty acid removal occurs slowly - approximately half were removed within 15

hours, and 3 days were required for complete deacylation [109]. Ultimately, the released fatty acids may be metabolised, excreted or incorporated into cellular lipids [119,121].

Macrophages are also capable of removing the lipid-A associated phosphates - both of which are critical for efficient TLR4/MD2 triggering [125]. Like deacylation, this is a slow process - murine peritoneal macrophages release about two thirds of lipid-A associated phosphate within 48 h [125]. However, LPS may also be dephosphorylated extracellularly, particularly via alkaline phosphatases (AP), which are non-specific hydrolases for phospho-monoesters expressed most abundantly in the intestine and lung [136-138]. The intestinal isoform of AP (IAP) is expressed throughout the small intestine, where it is localised to the apical brush border and secreted surfactant-like particles [139]. Cryostat sections of intestinal tissues show that IAP is able to dephosphorylate both rough and smooth forms of LPS [137], yielding a mono-phosphoryl lipid-A which possesses approximately 100-fold lower biological activity than canonical bisphosphoryl lipid-A [33,136,138]. The primary physiological function of IAP is not clear, but its inhibition increases susceptibility to endotoxaemia and reduces survival in Gram-negative sepsis in rodents [136,137], and promotes intestinal inflammation in Zebrafish [140]. Mice genetically deficient in IAP develop increased circulating levels of LPS, obesity and insulin resistance, in an IAP-supplementation-reversible manner [141].

Together, deacylation and dephosphorylaton result in lipid A structures with greatly reduced or no TLR4/MD2 agonist activity (as summarised in Figure 1B). However, as the degradation of LPS by the liver remains incomplete even after several days or weeks [112,121], the dominant mechanisms of suppression of inflammation in the acute setting are more likely dependent on sequestration and tolerance.

9) Limitations of the limulus assay

The limulus assay is based on the discovery by Levin and Bang that the blood of the horseshoe crab *Limulus polyphemus* coagulates in the presence of endotoxin [142]. This process is mediated by a proteolytic zymogen cascade, beginning with Factor C, which detects and is activated by endotoxin, to cleave Factor B, which in turn activates a pro-clotting enzyme. These factors are abundant in the granules of the crab's amoebocytes, which are commercially harvested to prepare a lysate which forms the basis of most limulus assays. Endotoxin causes the cleavage of coagulogen in the lysate to self-assembling coagulin, which results in a measurable increase in turbidity and eventually the formation of a gel clot, both of which have been used to detect endotoxin. However, the most widely used version of the assay today employs a chromogenic peptide, which when cleaved by the activated clotting enzyme releases a coloured mediator, typically p-nitroaniline, with peak absorbance around 405-410 nm. This variant of the assay may be read either at a single timepoint (as an endpoint assay), or at regular intervals (as a kinetic assay), where the time taken to reach a specific absorbance above baseline is used to determine bioactivity. Notably, the crude amoebocyte lysate also contains the zymogen Factor G, which is activated by glucan to promote the same pathway. Thus, most variants of the limulus assay are sensitive to both endotoxin and glucan (Figure 3).

As described above, the association between plasma endotoxin levels and risk of metabolic diseases has been driven almost entirely by measurements made using the LAL assay. However, this assay faces a number of key limitations that should be borne in mind when drawing conclusions from such studies. The first major factor to consider is that while the limulus assay is sensitive to low concentrations of LPS (the lower limit of sensitivity is around 1 pg/ml or 0.01 EU/ml *E. coli* LPS), it is also highly susceptible to false-positives arising from environmental contaminants. Because endotoxin is ubiquitous in the environment (especially in water and on surfaces exposed to dust), exhaustive care is required

to avoid unintentional contamination of the assay with exogenous LPS. One of the most frequent sources of contamination is water from laboratory stills or reverse osmosis systems, which in our experience almost invariably contain high levels of LPS, presumably arising from internal biofilms. Another common source of contamination is heparin, which is prepared commercially from pig intestines. Indeed, heparin-coated tubes for blood collection were shown to frequently contain large quantities of LPS, as did plastic vacutainers [143]. In our experience, the only blood collection tubes that reliably do not introduce significant contamination are glass vials, containing citrate for anti-coagulation if required.

Next, just as the rapid insertion of LPS into lipoproteins masks it from detection by the innate immune system, so this process also renders LPS invisible to the enzymes of the LAL assay [78,79]. Most attempts to measure plasma LPS therefore include a step of heat-treatment (typically 70^{o}C for 10 minutes) and dilution (typically 1:10) in pyrogen-free water, since it was shown that the inhibitory properties of serum are abolished if treated in this way before the addition of LPS [94]. However, this procedure only releases inhibition if it is performed before the LPS is added to blood [31,94]. The recovery of LPS from blood after it has bound to lipoproteins is far more challenging. It has been suggested that re-extraction of plasma using chloroform could be used to release LPS from inhibitory proteins and lipoproteins [94]. However, although this method successfully recovers LAL-reactive LPS after co-incubation with LDL, only a very small proportion (~0.001%) of the spiked biological activity is recovered [78]. Thus, it is likely that the majority of LPS in the circulation residing in lipoproteins is not detectable by the LAL assay, even after "de-masking" by commonly reported methods involving heat-treatment and dilution.

Interference is another confounder that needs to be considered. In particular, most commercially available variants of the LAL assay are activated by β-glucans, which are β-D-glucose polysaccharides found in the cell walls of cereals, yeast, bacteria, and fungi. Notably,

because they are abundant in the diet and may be absorbed from the intestinal lumen [144], β-glucans are present in the human circulation (~11 pg/ml in healthy volunteers) [145]. This raises the possibility that at least some of the reported increase in endotoxaemia post-prandially may reflect absorption of dietary β-glucans. Interference can also arise from the similarity in colour of serum or plasma with the peak absorbance of p-nitroaniline, the most often used the readout for the LAL assay. This is particularly problematic when the endpoint (single timepoint) assay is used. Another common confounder is serum concentration of triglycerides, which also promote absorbance at 405-410 nm. This specific issue is frequently revealed as a strong correlation between measured concentrations of LPS and triglycerides in epidemiological studies. For example, in a study of type II diabetes patients, these two variables were strongly correlated ($P<0.0001$), with a coefficient of regression of $r=0.65$ [12]. Post-prandial lipaemia, is therefore also likely to be a confounder of studies of post-prandial endotoxaemia, most readily addressed by use of the (background corrected) kinetic assay.

A final point that should be considered with respect to the suitability of the LAL assay is that the specificity of its lipid-A receptors can be quite different from that of human TLR4/MD2. For example, the synthetic lipid A analogue E5564, which is a potent inhibitor of TLR4/MD2, is an activator of the LAL assay [146]. Likewise, penta-acylated *Bacteroides* LPS, which is by far the most abundant type of LPS in the human large intestine, is an activator of the LAL assay, but is an antagonist of human TLR4/MD2 [44,147]. Thus, if metabolic endotoxaemia does occur, the limulus assay will likely fail to identify whether the LPS is of neutral, pro- or anti-inflammatory impact in human systems [59].

10) Alternatives to the limulus assay

These limitations have led to the development of numerous alternative approaches to the measurement of LPS in plasma or serum. Although a comprehensive list of these alternatives

is beyond the scope of this review, almost all fall into the categories of those based on LPS-specific antibodies, limulus-related proteins, chemiluminescent detection of neutrophil activation, mass-spectrometry for lipid-A-specific fatty acids or indirect measurement through depletion of LPS inhibitors. The relative merits of these approaches are discussed below, and summarised in Table 1.

Antibodies targeting the O-polysaccharide of LPS have been used to develop ELISA- and competitive radioimmunoassay-based methods for LPS quantitation [84,148]. Antibodies have also been used to immobilise LPS to plates for detection by LAL or related LPS-binding enzymes (the basis of the so-called 'Endolisa' and 'immunolimulus' assays) [149]. However, although these variants are reasonably sensitive and can be less prone to interference from sample colour, turbidity or glucans due to intermediate washing steps, a key limitation of such assays is that the O-polysaccharide of LPS is highly variable between species and strains (*E. coli* alone has over 150 distinct O-serotypes [34]). Thus, assays dependent on the use of antibodies are inherently limited to the detection of only those LPS bound by the antibody in question. Since there may be hundreds of different Gram-negative species in the intestinal lumen of a human volunteer, and many more in the food they consume, it would be almost impossible to produce a cocktail of antibodies targeting each of these different O-polysaccharides. One potential means of progress is through the use of antibodies targeting the core saccharide regions, which are more highly conserved than the O-antigen [34,150]. For example, the core-specific antibody WN1-222.5 has been shown to bind to rough or smooth LPS of all *E. coli* core types (R1, R2, R3, R4 and K12) and a number of other enterobacteria [100]. However, it will likely not bind to core saccharides from most organisms outwith the Enterobacteriaceae [100,151].

Antibodies are also used as the basis for the so-called Endotoxin Activity Assay (EAA). In this approach, the production of immune complexes between LPS and specific antibody is

detected via the induced respiratory burst of neutrophils in a blood sample [152]. Specifically, whole blood is mixed with antibody, complement opsonized zymosan is then added to trigger neutrophil activation and the resulting production of reactive oxygen species is measured by chemiluminescent assay [152]. The monoclonal antibody E5 is used, which is said to target the well-conserved lipid A domain of diverse bacteria [153]. However, this antibody has been widely criticised for its low affinity for LPS and its non-specific binding to diverse hydrophobic molecules, including several of host origin [154-156]. Thus, although the EAA has received US Food and Drug Administration approval for the detection of LPS in human blood samples, it is subject to the same limitations as other antibody-based assays.

Mass-spectrometry (MS)-based methods offer, in theory, a means for the unequivocal identification of LPS in plasma, since the lipid-A domain is both well-conserved and unique to LPS. Early studies, aiming to quantify the 3-OH fatty acids of lipid A (since it was assumed that their presence in blood would arise solely from lipid A), showed some promise. Plasma 3-OH 14:0 was 50-fold higher in rats injected with *E. coli* LPS than in saline-treated controls [157]. GC-MS for 3-hydroxy lauric acid as a specific marker of neisserial lipid A also correlated well (r=0.98) with measurement of LPS using the LAL assay in sera of 7 patients with fulminant meningococcal septicemia - a condition characterised by extremely high (in cases >200 ng/ml) plasma LPS levels [6]. However, when plasma samples from healthy animals or human volunteers were assayed in the same way, low levels of diverse 3-OH FAs were routinely detected [6,157,158]. Moreover, equivalent concentrations of 3-OH FAs ranging from 10 to 18 carbons in length were detected in blood from both conventional and germ-free rats [159]. Together, these findings suggest that the majority of 3-OH FAs in human blood arise not from lipid-A, but from mitochondrial beta-oxidation of fatty acids, thus precluding the use of 3-OH detection as a means to quantify low-grade endotoxaemia [157,159]. However, LC/MS/MS for intact lipid A molecules has been shown to be feasible,

as it was used successfully to measure plasma concentrations of the lipid A analogue E5564 in clinical trials [67,69]. In principle, this approach would be superior to 3-OH measurement, since it would yield not only definitive proof of lipid A, but also some idea as to whether or not the detected lipid A species are agonists for TLR4/MD2. The present limitation is sensitivity - the quantifiable limit of ~5 ng/mL is approximately three orders of magnitude higher than the expected level of LPS in the normal human circulation [67]. Nevertheless, as the sensitivity of mass-spectrometers improves, this method offers promising future development.

Finally, approaches have been developed to seek indirect evidence of recent endotoxaemia by monitoring the depletion of LPS inhibitors from blood. The so-called 'Endotoxin Neutralisation Capacity' (ENC) assay is often used for this purpose, by measuring the capacity of untreated plasma or serum to inhibit the bioactivity of a relatively large quantity of LPS (e.g. 20 ng/ml) in the LAL assay. Plasma ENC has been shown to be reduced in patients with alcoholic liver disease and to decline with time in patients attending for major elective surgery or colonoscopy [92,160,161]. This loss of ENC is considered to be a marker for low-grade transient endotoxaemia which may be missed by conventional LAL testing [92,160,161]. Plasma ENC values vary greatly between healthy volunteers, and appear to be associated with protein levels of ApoB, but not total IgA, IgG, IgM, alpha 2-macroglobulin, transferrin or ApoA [162]. Antibodies targeting the core epitopes of enterobacterial LPS (so-called EndoCAb antibodies) are present in almost all human sera [163,164], and these were found to correlate strongly with ENC in sera of 203 patients undergoing cardiac surgery, suggesting a major role for such antibodies in LPS neutralisation [102]. Thus, the use of ELISA to measure depletion of EndoCAb titre has also been suggested as a means of detecting episodes of transient endotoxaemia [92,160,161]. However, because low-grade contamination of reagents with endotoxin is almost ubiquitous, the possibility remains that

many of the reports of associations between plasma LAL bioactivity and disease might instead merely reflect an inverse association with ENC.

11) Relevance of LPS pharmacokinetics to studies of metabolic endotoxaemia

Together, the results of the studies reviewed above paint a complex picture of the likely fate of LPS which has been absorbed from the gut (as summarised in Figure 4). The majority of lumenal LPS is normally present, with the bulk of the endogenous microbiota, in the large intestine. However, the small intestine, with its much larger surface area, permeability to antigens and absorptive capacity for lipids, is thought to be the major site of LPS absorption *in vivo* [26,165-168]. That which translocates via the transcellular route is thought to be packaged by enterocytes into nascent chylomicrons, which traffick to the circulation via the lacteal and thoracic lymphatic duct. Studies using radiolabelled LPS and immunogold staining suggest that at least some of these LPS-chylomicron complexes escape clearance by the mesenteric lymph nodes to reach the blood [26,169]. By contrast, that portion which transits via the paracellular route is likely delivered at least in part to the portal blood, which reaches the liver within seconds.

LPS that has bound to chylomicrons will likely be removed from the circulation over several hours by ApoB and ApoE receptors (e.g. LDLR and LRP-1) expressed by hepatocytes, or via other receptors if re-distribution to other lipoprotein classes LPS has occurred [74]. That which enters via the paracellular route likely arrives in the form of micelles or aggregates. Most of this free LPS will be cleared from the circulation rapidly by Kupffer cells via scavenger receptor uptake, perhaps also by Fc-receptor mediated uptake if O-antigen-specific antibodies are present [118,119]. The remainder will be rapidly intercalated, with the help of lipid shuttle proteins such as LBP, PLTP and sCD14, into lipoprotein particles (LDL, VLDL and HDL) and effectively neutralised by sequestration of

the bioactive lipid A domain within the phospholipid monolayer [78,86,88]. These complexes may then circulate with a half-life of a dozen hours or so, being eventually cleared by hepatocytes via apoprotein receptors [66,86,115,116].

The extent to which lumenal LPS translocates into blood in health is perhaps the most critical question in this debate, but a consensus on this point is yet to emerge. In particular, studies employing the limulus assay, or clinical indicators of endotoxaemia, to measure LPS absorption after oral administration of the substance to healthy animals have yielded mixed results [26,166,168,170-176]. Nevertheless, a more consistent picture has emerged from studies measuring translocation of radiolabelled antigens. For example, rats orally gavaged with a ^{14}C-labeled *E. coli* extract showed translocation of antigens up to 30 kDa in mass to plasma within 4 h, and the majority of those studied remained immunologically intact, suggesting little degradation during transit [177]. Likewise, ~2% of the radiolabel derived from ^{32}P-labelled *E. coli* extracts administered orally to rabbits appeared in liver within 8 hours [178]. Crucially, approximately half of the translocated label was shown to be LPS using O-antigen-specific immunoassays [178]. In rats, ~0.023% of orally delivered ^{3}H-labelled LPS was found in plasma 90 minutes after gavage (increasing to 0.12% following surgically impaired bile production) [173]. In mice, ~0.1% of orally delivered ^{125}I-LPS is found in blood 6.5 hours after oral gavage, increasing to 0.25% after stimulation of chylomicron production with triolein [26]. Thus, the available evidence suggests that a considerable proportion of ingested LPS is likely to reach the circulation, and eventually the liver.

Most of the LPS which is delivered to hepatocytes appears to be excreted to bile before any enzymatic degradation has occurred [112]. That which remains may be degraded slowly before excretion over the course of several days. By contrast, the fraction that is delivered in the first instance to Kupffer cells may be acted upon by phosphatases to remove lipid-A

associated phosphates, and AOAH to remove secondary fatty acids, so yielding detoxified LPS which may then serve a regulatory role as an endogenously produced antagonist of TLR4/MD2 (at least in man) [131-133]. Although it remains unclear how processed LPS is transferred from Kupffer cells to hepatocytes, it is possible that some may be transmitted via the observed "efflux" of LPS to HDL by ABCA-1, for eventual clearance by hepatocyte SR-BI [122,124].

This scheme suggests that numerous defences exist which protect the mammalian host against the inappropriate induction of inflammation by LPS arriving from the gut. First, the enzyme IAP, which is expressed by enterocytes of the small intestine, presumably detoxifies at least a proportion of ingested LPS [140,141]. The contiguous tight junctions of the enterocyte layer then prevent most LPS from gaining access to lymph or blood. Next, the low level of expression of TLR4 accessory proteins by enterocytes and the generally anti-inflammatory milieu of the gut mucosa limit responses to LPS which may translocate to reach underlying cells. From here, the LPS that is incorporated into chylomicrons in lymph, or LDL/HDL in plasma, will likely be sequestered from TLR4 all the way from gut mucosa to hepatocytes, which lack (in health) expression of the MD2 and CD14 proteins required for LPS responsiveness [179]. The LPS which remains in the free form and is therefore able to activate macrophages or circulating monocytes is rapidly cleared from the circulation by Kupffer cells. Notably, in health at least, Kupffer cells are relatively unresponsive to LPS compared to other types of macrophage [180].

Another potentially significant defence is antibody, which, via IgA, may act to repel LPS entry from the lumen or neutralise its bioactivity in serum and promote FcR-dependent clearance via IgG and IgM [104,118]. However, this defence is dependent on the presence of antibodies targeting the O-polysaccharide of LPS, which are raised naturally to commensal organisms, but may or may not cross-react with LPS of food-borne bacteria [118,119].

Clearly, if the proportion of ingested LPS that is absorbed in man is of the same order observed in the animal studies cited above, then the majority of the absorbed LPS must be neutralised, detoxified or rapidly cleared, since processed meats can contain >1 mg LPS per serving [181]. Thus, it seems likely that in health, these defensive mechanisms enable the accommodation of low-grade entodotoxin translocation with little or no induction of inflammation systemically.

12) Conclusions

The demonstration of remarkable alterations to lipid and glucose metabolism in human and animal models of endotoxaemia has fuelled much interest in the potential of LPS to drive chronic metabolic disease. However, despite decades of measurements using the limulus assay, a consensus is yet to be reached on the true range of circulating LPS concentrations in health, how much of this is driven by metabolic endotoxaemia and whether such levels may impact on human health. Indeed it remains possible, as a consequence of the numerous difficulties inherent to the LAL assay described above, that many of the reported associations between serum LPS levels and disease reflect confounders, such as triglyceride levels, or the unintentional measurement of ENC through exposure of samples to ubiquitous contaminants at time of collection or assay.

Clearly, in order to make progress in addressing these questions, new assays for endotoxin measurement are required. Whatever form these may take, they will need to be able to counter the four key limitations faced by current iterations of the LAL assay. First, they will need to incorporate a robust recovery step that counters the interference from protein and lipoprotein binding. Second, they will need to be able to detect the sequestered but biologically active lipid A component of the molecule, rather than the exposed but variable saccharide domains, which effectively precludes the use of antibody-based techniques. Third,

the assays must be resistant to false positives induced by unrelated molecules. And finally, measurements of biological activity must reflect the activity of those lipid A structures that activate human TLR4/MD2, which may not be reflected by invertebrate PRRs (such as the LAL assay enzymes).

Nevertheless, an increasing body of evidence from a wide variety of approaches, particularly *in vivo* disease models, suggests that endotoxin is likely to play a key role in the development of several metabolic conditions. But it is also becoming apparent that we have effective defences against the inappropriate induction of inflammation by LPS arriving from the gut. These mechanisms are synergistic and interconnected, including enterocyte barrier function, IAP, AOAH, O-antigen-specific antibodies, scavenger receptor based clearance and sequestration by circulating lipoproteins. If metabolic endotoxaemia is identified as a therapeutic target in future, an improved understanding of these defences could lead the way to new approaches for the prevention or treatment of diverse metabolic diseases.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure legends

Figure 1: Structures of LPS and lipid A of major commensals of the human intestinal tract

(A) The major functional domains of LPS molecules are conventionally designated lipid-A, inner core, outer core and O-polysaccharide (O-antigen). (B) Structures of canonical *E. coli* and *B. fragilis* lipid A are shown. Note the mono-phosphoryl, penta-acylated structure of *B. fragilis* lipid A, which is thought to account for its lack of agonist activity with respect to human TLR4/MD2. Highlighted elements of *E. coli* lipid A indicate those parts of the molecule which may be removed by intestinal alkaline phosphatase (IAP) and acyloxyacyl hydrolase (AOAH) during the detoxification process.

Figure 2: Kinetics of LPS bioactivity and clearance from the circulation following intravenous injection

(A) This figure, prepared from data presented by van De Venter *et al* [29], shows the bioactivity of LPS as measured using the limulus assay, in human plasma following a bolus dose of 2 ng/kg LPS. (B) This figure, reproduced with permission from Coulthard *et al* [30], shows the biphasic clearance of smooth (S-) or rough (R-) type ^{3}H-radiolabelled LPS following intravenous injection in mice. Note that the x-axis (time) is not linear.

Figure 3: Schematic representation of the LAL assay

The key mediators of the proteolytic zymogen cascade underpinning the LAL assay are shown. Activity of the clotting enzyme, which is related to the quantity of LPS in the reaction, is typically measured by monitoring cleavage of a chromogenic peptide. Both rate of change of product and end-point absorbance are used for LPS quantitation.

Figure 4: Mechanisms of LPS clearance from the circulation

LPS entering from the gut is likely to enter via lymphatic drainage of lacteals, where the majority will likely bind rapidly to chylomicrons (CM), or venous capillaries leading to the portal vein, where a proportion will bind to lipoproteins and the remaining free LPS will be taken up by Kupffer cells via scavenger receptors (ScR) or immunoglobulin Fc-receptors (FcR), if O-antigen specific antibody is present. A proportion of LPS internalised by Kupffer cells may be detoxified by phosphatases and AOAH. Processed and intact LPS may then be "effluxed" via ABCA-1 to HDL. Lipoprotein-bound LPS, which is effectively non-inflammatory through sequestration of the lipid-A domain from PRRs, may then be cleared slowly via apoprotein receptors expressed by hepatocytes, e.g. the LDL-receptor (LDLR), the LDLR-related protein-1 (LRP-1) and scavenger receptor BI (SR-BI). LPS delivered to hepatocytes is thought to be dephosphorylated and deacylated further, albeit slowly and to a limited extent, before excretion to bile, which occurs over several days. Key mediators of LPS defence, clearance or detoxification are highlighted in green, and those which may promote translocation or pro-inflammatory recognition of LPS are shown in red.

Tables

Method	Advantages	Limitations
Limulus assay	• Inexpensive • Easy to use • Widely cited • Rapid generation of results	• Confounded by light scattering agents, such as triglyceride-rich lipoproteins • Does not reflect biological activity of LPS with respect to human PRRs • Prone to inhibition by lipid-A-binding agents, such as albumin and lipoproteins • Glucans, which are present in serum, give false positive readings
ENC assay	• Inexpensive • May reveal recent, transient exposure of an organism to circulating LPS that is missed by direct LPS measurement	• Confounded by any agent that inhibits LPS activity in the LAL assay (e.g. lipoproteins) • Does not reflect biological activity of LPS with respect to human PRRs • Glucans, which are present in serum, give false negative readings
ELISA	• Easy to use • Can offer high sensitivity • Rapid generation of results	• Depends on LPS-binding antibodies, which are specific for only a small fraction of naturally

	• Less prone to inhibitors	occurring LPSs • Will fail to detect LPS not targeted by chosen antibodies • Does not reflect biological activity of LPS with respect to human PRRs
EAA assay	• Can offer high sensitivity • Less prone to inhibitors	• Depends on LPS-binding antibodies, which are specific for only a small fraction of naturally occurring LPSs • Does not reflect biological activity of LPS with respect to human PRRs • Will fail to detect LPS not targeted by chosen antibodies • Requires fresh human blood
Mass-spectrometry	• Avoids use of limulus enzymes or antibodies • Potential for definitive identification of lipid A species • Less prone to inhibitors	• Insufficient sensitivity to detect lipid A in serum • Detection of 3-OH fatty acids is possible, but confounded by their abundance in serum due to fatty acid oxidation by host cells
Biosensor-based approaches	• Potential for rapid generation of results	• Most are based either on antibodies, limulus enzymes or similar proteins for LPS capture, so lack range and the ability to predict LPS bioactivity with respect to

		human PRRs
Cell-based assays (including cultured macrophages and HEK-293-TLR4 transfectants)	• Can accurately reflect biological activity of LPS with respect to human PRRs • Can be used to identify LPSs which are antagonist or anti-inflammatory in man	• Requires culture of living cells • Requires dilution of sample in tissue culture medium, so lowering sensitivity • Relatively time consuming and expensive compared to other methods • May be inhibited by LPS-binding agents

Table 1: Comparison of methods for the quantitation of LPS in serum