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Early View

Review

Human lipopolysaccharide models provide mechanistic and therapeutic insights into systemic and pulmonary inflammation

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HUMAN LIPOPOLYSACCHARIDE MODELS PROVIDE MECHANISTIC AND THERAPEUTIC INSIGHTS INTO SYSTEMIC AND PULMONARY INFLAMMATION

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ABSTRACT

Inflammation is a key feature in the pathogenesis of sepsis and acute respiratory distress syndrome (ARDS). Sepsis and ARDS continue to be associated with high mortality. A key contributory factor is the rudimentary understanding of the early events in pulmonary and systemic inflammation in humans, which are difficult to study in clinical practice, as they precede the patient's presentation to medical services. Lipopolysaccharide (LPS), a constituent of the outer membrane of Gram-negative bacteria, is a trigger of inflammation and the dysregulated host response in sepsis. Human LPS models deliver a small quantity of LPS to healthy volunteers, triggering an inflammatory response and providing a window to study early inflammation in humans. This allows biological/mechanistic insights to be made and new therapeutic strategies to be tested in a controlled, reproducible environment from a defined point in time. We review the use of human LPS models, focusing on the underlying mechanistic insights that have been gained by studying the response to intravenous and pulmonary LPS challenge. We discuss variables that may influence the response to LPS before considering factors that should be considered when designing future human LPS studies.

Keywords: LPS, endotoxin, inflammation, human models, sepsis, ARDS.

INTRODUCTION

Systemic inflammation is central to the pathogenesis of sepsis, which carries a mortality of up to 25% [1]. Acute respiratory distress syndrome (ARDS), which has a mortality of 2-40%, is characterised by acute pulmonary neutrophilic inflammation accompanied by systemic inflammation [2].

Characterising sepsis and ARDS is hampered by incomplete understanding of the pathogenesis of acute systemic and pulmonary inflammatory responses in humans. Clinically, the earliest host responses can rarely be studied, as patients develop illness before presentation. Animal (particularly rodent) models provide some insight but there are differences in the immune responses in human and rodents [3].

Bacterial lipopolysaccharide (LPS, or endotoxin) is a virulence factor of the outer membrane of Gram-negative bacterial cell walls (Figure 1) that elicits an inflammatory response [4] (Figure 2). Experimental administration of LPS to humans allows access to the initial inflammatory response and how it evolves, thus allowing mechanistic insights into human inflammation to be made. Understanding the mechanism of the response helps to identify potential therapeutic targets.

In this article we provide an overview of different human LPS models including the temporal inflammatory changes induced, limitations and challenges, and future considerations for ongoing human LPS studies. Extensive literature around the role of LPS in pulmonary/occupational lung pathology [5] or inhaled LPS models in smokers and atopic patients [6, 7] will not be considered.

Types of Human LPS Model

LPS models vary in their route of delivery and the inflammatory response generated. A summary of the source and dose of LPS used in the studies referenced in this review is available in Table 1.

Intravenous LPS triggers production of cytokines and trafficking of leukocytes from and to the systemic circulation [8]; doses of IV LPS vary between studies from 0.06-4ng/kg. The response is dose-dependent: [9] 2ng and 4ng/kg doses have been used to induce a more pronounced inflammatory response (2ng/kg has been validated as eliciting a systemic inflammatory response syndrome [8]), whereas lower doses are used to model conditions where chronic, low-grade inflammation plays a role, such as type 2 diabetes [10].

Respiratory LPS models exist in two forms; inhaled challenge uses a nebulised saline solution containing LPS, aiming to induce a diffuse pulmonary inflammatory response. Alternatively, LPS can be instilled bronchoscopically, producing a localised response in a bronchopulmonary segment, simulating the inflammatory response in bronchopneumonia. Pathophysiologically, LPS-induced chemokines drive recruitment of activated neutrophils to the lungs, with leak of protein-rich fluid across the alveolar-capillary membrane [11], similar to that seen in the hyperinflammatory endotype of ARDS [12].

Dose comparisons between respiratory models are difficult; in contrast to IV models, where LPS used is almost uniformly NIH Clinical Centre Reference Endotoxin

(CCRE), derived from *Escherichia coli* 0113, many respiratory studies use 20-60μg of other commercially available preparations (see Table 1).

Whilst respiratory challenge models provide tissue specific information, repeated sampling and analysing the stromal response remains challenging. As such, nasal models, whereby LPS is applied to the mucosa followed by lavage at pre-determined time points have been developed. These provide an alternative means of analysing local inflammatory responses with less invasive sampling [13]. Doses vary from 10-100μg and tend to use non-CCRE commercial preparations [13–15]. Finally, intra-dermal injection of CCRE grade LPS has been used as a model to gain insight into the localised inflammatory response in tissue and is the only model that allows detailed insights into the stromal response [16].

LPS structure and chemistry

The LPS molecule is found in the outer membrane of Gram-negative bacteria and is comprised of an O-specific side chain, a core region and lipid A molecule [4, 17, 18] (Figure 1). The O-specific side chain is composed of oligosaccharide units attached to the outer region of the carbohydrate core [4,15]. The inner core contains both heptose and 2-keto-3-deoxyoctonic acid (KDO) and is joined to lipid A by a ketosidic bond [4, 17, 18]. Lipid A is composed of a phosphorylated glucosamine disaccharide backbone with fatty acid chains attached [4, 17, 18]. Lipid A is the immunologically active component of LPS.

LPS stimulates an inflammatory response by binding to cells expressing Toll-like receptor 4 (TLR4). Circulating LPS binds LPS-binding protein (LBP) [19] which is

recognised by the membrane-anchored co-receptor, CD14 [19]. This allows LPS to bind to a membrane protein, MD-2, and the extracellular portion of TLR4, which activates two signaling cascades [19, 20]. The Toll-IL-1-resistance (TIR) domain recruits adaptor proteins MyD88-adaptor-like protein (Mal) and MyD88, which allows activation of several IL-1 receptor-associated kinases (IRAKs). These IRAKs allow for ubiquitination of the adaptor molecule TNF-α receptor-associated factor 6 (TRAF-6) which activates the transforming growth factor beta-activated kinase 1 (TAK1) [20–22]. TAK1 phosphorylates several kinases, freeing their respective transcription factors NF-kappaB, cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1) to translocate into the nucleus [20–22]. In the second pathway, TLR4 is transported to an endosome where it recruits two more adaptor proteins; TIR domain-containing adaptor protein inducing IFN-β (TRIF) and TRIF-related adaptor protein (TRAM) [19, 20, 22]. This complex can recruit TRAF6 and stimulate NF-kappaB nuclear translocation through TAK1. TRIF ubiquitinates TRAF3, allowing it to recruit two kinases, TANK-binding kinase 1 (TBK1) and IKKi, which phosphorylate interferon regulatory factor 3 (IRF3) [20, 21]. IRF3 translocates to the nucleus and stimulates the production of type I interferons [20–22]. A graphical summary of the LPS signalling pathway is found in Figure 2.

METHODS

References in this narrative review were selected by a Pubmed search using the terms ‘LPS’, ‘lipopolysaccharide’, ‘endotoxin’, ‘endotoxaemia’ and ‘endotoxemia’ from 1990. Results were screened for investigation of biological/clinical endpoints, mechanistic insights or therapeutic agents and those considered by the authors to be

most relevant for building a comprehensive oversight of the effects of LPS in humans were selected for inclusion.

Values for Figures 3 and 4 were obtained from either graphs or the body of text from papers identified in the search strategy. These values were used to calculate a mean for each time point and plotted to create a summary display.

INTRAVENOUS LPS MODEL

Clinical parameters

The clinical response appears within an hour and begins with headache and myalgias, followed by a tachypnoea 2-3 hours later [8]. Core temperature rises by 1-3°C with maximal increases seen 3-4 hours post-administration [8, 23–27], which coincides with a tachycardia of ~90-100bpm [8, 23, 24, 26–29]**.** A small decrease in mean arterial pressure is observed by 3 hours [26, 29–31]. Temperature and blood pressure normalise by 24 hours, whereas heart rate remains mildly elevated [8, 28].

LPS exerts complex cardiovascular effects characterised by a hyperdynamic state but with reduced ventricular contractility. Left ventricle function is impaired relative to saline-loaded controls, and load-independent contractility indices are reduced. This implies LPS enhances cardiac output through decreased systemic vascular resistance [29–32]. There are reports of transient bradycardic episodes occurring in fasted volunteers following LPS exposure [33, 34]. This is an exaggerated Bezold-Jarisch reflex (a triad of bradycardia, vasodilation and hypotension resulting from stimulation of cardiac receptors as a homeostatic response to hypovolaemia) in a setting of high vagal tone [35]. Many researchers try to prevent this by hydrating

volunteers with isotonic crystalloid solutions, this increases intravascular volume but can attenuate the inflammatory response [36].

Haemostatic parameters

Activation of the coagulation system is demonstrated by increased levels of fibrin [37] and tissue factor [38, 39] at 2 hours, followed by rises in plasma prothrombin fragments [40–47] and thrombin-antithrombin (TAT) complexes [41–45, 48, 49] , which peak at 4 hours. This haemostatic response is unaffected by inhibition of IL-6 [45].

Changes in coagulation are followed by upregulation of fibrinolysis, with plasmin-antiplasmin (PAP), tissue plasminogen activator (tPA) and d-dimer levels peaking at 2, 3 and 4 hours respectively [41–43, 46, 48, 49]. By 4 hours, levels of plasminogen activator inhibitor-1 (PAI-1) rise, indicating a switch to fibrinolytic inhibition [41–43]**.** Coagulation and fibrinolysis are susceptible to modulation, and both are inhibited by IL-10 [31]. Adrenaline inhibits coagulation but enhances fibrinolysis, demonstrating anti-thrombotic effects in the presence of LPS [50]. All haematological parameters return to baseline by 24 hours.

Cellular parameters

Circulating neutrophils initially fall, with a nadir at 1 hour, rising to maximal levels around 4-6 hours [24, 25, 43, 51]. This can be attenuated by co-administration of IL-1 receptor antagonist [52]. Granulocyte colony-stimulating factor (G-CSF) concentrations peak around 4 hours, suggesting neutrophils are mobilised from bone

marrow [25, 27, 51]. Absolute monocyte and lymphocyte counts fall, reaching lowest levels between 1 and 3 hours [9, 24, 53]; both return to baseline by 24 hours.

Molecular changes and LPS tolerance

Epigenetic changes have been described *in vitro* 1 hour after LPS exposure in monocytes; they result in promotion of the pro-inflammatory response and inhibition of differentiation [54, 55]. Histone modifications result in an open chromatin architecture and increased transcription of genes potentiating the inflammatory response [54, 56–58]. Nucleosome remodelling [56], DNA methylation and micro-RNAs also play a role in the transcriptional response to LPS in macrophages *in vitro* [56, 58].

By 2 hours, macrophage expression of transcription factors (TFs) *in vitro* has changed significantly [59]. *In vivo,* the transcriptional response to LPS can be divided into early up-regulated (such as those coding for key inflammatory mediators), late up-regulated (anti-inflammatory products, apoptosis) or down-regulated genes (bio-energetic processes like oxidative phosphorylation) [60–62]. Recently, one group has demonstrated that the transcriptomic response to LPS is dose dependent, identifying 3736 genes differentially regulated by dose. Multivariate modelling showed up-regulated genes again included those coding for TLR4 signalling and interleukin production as well apoptosis, with down-regulated genes coding for lymphocyte signalling pathways. Univariate analysis revealed that nearly 40% of the variance in the dose-dependent gene set was due to changes in leukocyte counts [63]. These findings again highlight that the response to LPS is dose-dependent.

Furthermore they show that transcriptomic analysis may be used to unmask subtle variations in LPS response which have previously gone undetected.

Metabolomic analysis of plasma reveals that at 6 hours, lipid metabolism is upregulated whilst protein metabolism is suppressed; by 24 hours, lipid metabolism is downregulated and amino acid metabolites are upregulated [64]. In one study, 15 of 16 significantly altered metabolites had similar changes in IV LPS-challenged individuals and septic patients [65]. Additionally, septic patients who survived shared directionality in 18 of 20 differentially regulated metabolites at 24 hours with LPS-exposed subjects, compared to 9 of 20 for non-survivors [65], suggesting that, LPS models mimic the “controlled response leading to recovery” phenotype in sepsis.

Tolerance to LPS, whereby cells exposed to repeated doses display a diminished capacity to generate an inflammatory response [66] is an important feature of IV LPS models both in terms of experimental design and in providing insights into immunosuppression in critical illness. *Ex vivo* and *in vivo* tolerance have different kinetics; *ex vivo* tolerance appears rapidly, by 3 hours [67], and resolves within 7 days, whereas *in vivo* tolerance persists for at least two weeks [68], abating by 5 weeks [69].

In vitro models demonstrate tolerance is accompanied by enhancement of inhibitory intra-cellular signalling pathways (e.g. p53 signalling), accumulation of transcriptional repressors, the absence of pro-inflammatory activators (e.g. STAT) at gene promoters [54] and down-regulation of TLR4 expression (*in vivo*) [70].

Biochemical and cytokine parameters

TNF-α [9, 27, 28, 71], interferon-gamma (IFN-γ) [72], IL-6 [9, 24, 27, 28, 51] and IL-8 [9, 24, 27, 51] reach peak levels within 1-2 hours and return to baseline after 3-6 hours (TNF-α and IL-6) [9, 24, 27, 28, 71] or 24 hours (IL-8) [9, 27]**.** Markers of neutrophil degranulation are seen within 6 hours [25, 26, 40, 42]. Several chemotactic mediators are also released, macrophage inflammatory protein (MIP-1α) [27], growth-related oncogene-alpha (GRO-α) [27, 28], monocyte chemoattractant protein (MCP-1) [42] and interferon gamma-induced protein (IP-10) [73] levels peak at 2-5 hours. Antecedent oral corticosteroids inhibit the rise in several inflammatory cytokines and chemokines [42], as does blocking $P2Y_{12}$ receptors [74].

LPS also triggers a compensatory anti-inflammatory response. IL-10 peaks at 3 hours [27, 42, 43, 51, 72] with IL-1 receptor antagonist (IL-1Ra) following at 4 hours [25, 27, 28, 42, 51]. Induced circulating soluble TNF-α receptor is highest between 2-4 hours [25, 27, 42, 75]. Several interventions aimed at manipulating the anti-inflammatory response have been trialled using IV LPS models. Adrenaline attenuates pro-inflammatory cytokine release [76] whilst enhancing levels of IL-10 [76, 77]. IL-10 release is also increased by the action of nicotine (which stimulates α7-nicotinic acetylcholine receptors, the end point of the vagal anti-inflammatory reflex), highlighting a role for the autonomic nervous system in modulating acute inflammation [78]. Dipyridamole augments anti-inflammatory cytokine secretion [79], which may help explain the association between anti-platelet therapy and improved survival in sepsis [80]. Allogenic adipose mesenchymal stem cell (MSC) infusion,

when delivered prior to LPS exposure, has immunomodulatory effects that could be beneficial in sepsis [81].

Following IV LPS, the vascular response to noradrenaline [82] is reduced. This is a result of oxidative stress and correctable with high doses of anti-oxidants [82, 83]. Intrinsic catecholamine production is increased however, with adrenaline levels rising by 1-2 hours [8, 27, 71]. Endothelial cell activation is triggered by TNF-α [43] and is indicated by maximal blood concentrations of vascular endothelial growth factor (VEGF) [40] and soluble thrombomodulin (sTM) [84] at 3-4 hours, von Willebrand factor (vWF) at 5-6 hours [26, 48] and E-selectin at 6-12 hours [25, 27, 43, 48, 84]**.** vWF and E-selectin levels are still raised at 24 hours [25, 43, 48].

Whilst there is no change in urea and creatinine clearance, renal plasma flow increases 3 hours after LPS infusion [85]. Evidence of localised damage is present; levels of cytosolic glutathione-S-transferase-A1 (GSTA1-1), a marker of proximal tubular damage, are raised between 6 and 12 hours [86]. Local formation of reactive oxygen and nitrogen species are thought to contribute to this damage, as iNOS mRNA levels isolated from urinary cells are significantly increased [86]. Novel biomarkers of early kidney injury, such as kidney injury molecule 1 (KIM1), are also increased by LPS [87]. Treating patients with sepsis/septic shock with alkaline phosphate infusions (which detoxifies lipopolysaccharide) improves renal function by inhibiting upregulation of iNOS and thus the formation of damaging reactive oxygen and nitrogen species [88].

Increases in acute phase proteins are evident at 24 hours; levels of LBP have increased 2-5 fold [89–91], serum amyloid-A (SAA) levels by at least 17-fold [92, 93], fibrinogen by approximately 30% [46, 94] and C-reactive protein (CRP) by at least two-fold [9, 24, 72].

RESPIRATORY LPS MODELS

Clinical parameters

Inhaled

Inhalation of LPS raises body temperature by 0.5-1.5°C, peaking 6-8 hours post-exposure [95, 96]. Flu-like symptoms such as headache and fatigue occur [97, 98]. Changes in blood pressure, respiratory rate or heart rate are uncommon [6, 95]. Significant heterogeneity in forced expiratory volume in 1 second (FEV_1) in healthy volunteers following LPS inhalation occurs [99, 100], which may explain why no significant change is demonstrated when evaluating interventions [95, 101–106]**.**

Instilled

Segmental LPS challenge produces minimal clinical symptoms. Some studies have observed a fall in arterial pressure 4 hours after exposure [107, 108]**,** decreased PaO_2 and increased $PaCO_2$ [108] or a reduction in FEV_1 [107]. Changes in heart rate are variable, with reductions reported at 4 [108] and 24 hours [107] but others finding no change [109, 110]. The most consistently reported changes are flu-like symptoms and increases in temperature of ~0.5°C by 6 hours [108, 109, 111–114].

Haemostatic parameters

Inhaled

At the alveolar level, soluble tissue factor and TAT complexes are found in increased concentrations in bronchoalveolar lavage fluid (BALF) at 6 hours [115, 116] whilst protein C activity is reduced [116]. Increased PAI-1 levels are also found, indicating local inhibition of fibrinolysis [115, 116].

Instilled

Instilled LPS results in raised levels of TAT, prothrombin fragments and soluble tissue factor, and depressed protein C levels, in BALF by 6 hours [117–119]**.** Elevated levels of tPA and PAP complexes demonstrate activation of the fibrinolytic system – this is followed by a rise in PAI-1, indicating fibrinolytic depression [117, 119].

Cellular parameters

Inhaled

Evidence of a cellular response is seen in induced sputum samples following LPS inhalation. The sputum neutrophil count peaks between 4-6 hours and remains elevated at 24 hours [95, 102, 120–122]*,* occurring as a result of p38 MAPK-stimulated [123] production of TNF-α, IL-1β and IL-8 [121, 122].

Lymphocyte levels are raised by 24 hours and normalise within 7 days [120, 121]**.** Macrophage levels are variably reported as either static [95, 102, 122] or increased [120, 121]. Biochemical evidence of cell activation mirrors increased cell counts, with the neutrophil enzymes myeloperoxidase (MPO), matrix metalloproteinase-9 (MMP9) and human neutrophil elastase (HNE) peaking at 6 hours [95, 104, 123, 124]**.**

There is a significant increase in BALF neutrophil count by 90 minutes [7], which remains elevated at 24 hours compared to placebo [106, 125, 126]. Long acting β-agonists impair alveolar recruitment of neutrophils through inhibition of adenylate cyclase [125]. Alveolar macrophage (AM) and lymphocyte counts probably do not increase [106, 125]. There is evidence of increased populations of blood derived monocytes and dendritic cells subtypes within the alveoli by 8 hours; these recruited cells rapidly develop similar gene expression profiles to native airway monocytic cells [127]. Interestingly, LPS is a factor in determining whether a Th1 or Th2 response is generated [128], with AM exposed to low doses of inhaled LPS (approximately 2μg), adopting a Th2 cytokine profile [129]. Inhalation of 60μg of LPS leads to the appearance of pulmonary monocyte-like cells (PMLCs) in the alveolar spaces [130] with reduced proliferative and phagocytic capacity compared to AM [131].

Systemically, a rise in peripheral circulating neutrophils is seen by 6-8 hours [95, 96, 101, 104, 120, 132] which persists to 24 hours [96, 101, 120]**.** The effect on monocytes is unclear; one study found a fall at 6 hours [95] and another, using a similar dose, found an increase at 8 hours [132]. Lymphocyte numbers are unaffected [95, 132]**.**

Instilled

A large increase in BALF neutrophil count occurs by 6 hours, which persists to at least 24 hours and is accompanied by smaller increases in macrophage and lymphocyte numbers [108, 113, 133]. Neutrophils recruited to the alveoli have a transcriptomic profile distinct from circulating neutrophils [134]. Instilled LPS results

in a peripheral blood neutrophilia with a decrease in the percentage of lymphocytes and monocytes [108, 110].

Biochemical and cytokine parameters

Inhaled

Levels of IL-6, IL-8 and TNF-α, stimulated through p38 MAPK signalling and subsequent IL-1β production [122, 123], peak at 6 hours in induced sputum samples [6, 95, 97, 123], as does secretion of the chemotactic marker MIP-1β [123].

In BALF, TNF-α is detectable at significantly elevated concentrations by 90 minutes and remains raised out to 24 hours [7, 105, 125, 126]**.** Levels of IL-1β [7] and IL-8 are also raised by 90 minutes, with IL-8 levels still high at 24 hours [7, 125, 126]. This is followed by an increase in IL-6 levels at 3-6 hours [105, 125]. Markers of increased neutrophil activity such as MPO and HNE peak at 6-9 hours [125, 132, 135]. Various chemoattractant molecules, such as MIP-1β and MCP-1 also rise by 6 hours [125, 136].

Systemically, IL-6 and IL-8 rise between 6 and 8 hours [122, 123, 132], with no change in TNF-α, IL-1β, IL-10 or MCP-1 [122, 123, 132]. Evidence of neutrophil activation is detectable systemically with HNE [132] and MPO [124] raised at 8 and 24 hours respectively. As with IV models, several strategies aimed at modifying the inflammatory response have been trialled using inhaled LPS. Statins reduce alveolar neutrophil recruitment and TNF-α, MPO and MMP levels [103], although did not improve outcomes in non-selected ARDS patients [137]. This is possibly because inhaled LPS may only model the hyper-inflammatory endotype of ARDS [138].

Recently, infusion of keratinocyte growth factor (KGF) prior to LPS inhalation has been shown to increase type II pneumocyte proliferation, suggesting it may promote lung repair [139].

Inhaled LPS also results in evidence of activation of endothelial cells locally, with raised levels of soluble thrombomodulin in BALF [116], and systemically, where circulating levels of soluble E-selectin rise by 24 hours [96, 104, 140].

By 24 hours a rise in acute phase proteins is seen systemically, with LBP increasing 2-5 fold [99, 104], fibrinogen increasing by approximately 30% [118] and CRP levels rising to between 2 and 10 times baseline values [104, 120]. Oral corticosteroids have been given in inhaled LPS models, reducing the rise in systemic CRP [104] (though with no effect on cytokine secretion [104, 121]).

No change in systemic haematological markers is observed [116].

Instilled

Both pro- and anti-inflammatory activity is detectable in BALF after segmental LPS instillation. TNF-α, IL-1β, IL-6, IL-8 and G-CSF peak at 6 hours [108, 111, 113, 133] and may remain significantly above baseline by 24 hours [108, 113]. IL-1Ra levels rise by 6 hours [108]; TNF receptor levels are also raised by this point and remain so at 48 hours [108]. ENA-78, lactoferrin, MPO and HNE are similarly elevated by 6 hours [108, 111]. Local secretion of chemotactic molecules occurs by 6 hours [108, 111]. By 12 hours, neutrophil-derived MMP-8 and β-defensin-2 concentrations are

increased [133]. This pattern of alveolar response parallels the hyper-inflammatory endotype in ARDS [138].

Evidence of endothelial activation following instilled LPS is variable, one study found soluble thrombomodulin in significantly raised quantities in BALF at 6 hours [117], whilst in another no increase was detected [112].

Systemically, there are significant increases in G-CSF, IL-6 and IL-1Ra over the 24 hours following LPS instillation, with CRP levels rising at 24 hours [108, 110]**.**

The development of tolerance is less clear in respiratory models, with alveolar macrophages exposed to bronchoscopically instilled LPS *in vivo*, producing higher levels of inflammatory cytokines upon subsequent *ex vivo* LPS stimulation than macrophages from the contralateral lung exposed to saline [141].

CHALLENGES AND LIMITATIONS OF HUMAN LPS MODELS

Most IV LPS models involve healthy young males given a single dose of LPS early in the morning. However, the response to IV LPS in healthy young males is not the same as other subjects. Females have higher levels of TNF-α [89, 142], CRP [89], cortisol [142], IL-6 [142] and a greater fall in mean blood pressure [89], whilst also avoiding the loss of sensitivity to noradrenaline that occurs in males [89] in response to LPS. Participation by older subjects is limited, but increasing age is associated with a prolonged fever response and more rapid rise in concentrations of TNF-α [143], and a greater fall in systolic blood pressure [144]. Body mass index (BMI) has

not been shown to affect the response to experimental LPS [145, 146], although the range of BMIs assessed has been limited.

Racial differences in the response also exist – Duffy antigen negative Africans have reduced thrombin formation and generate fewer TAT complexes, F1+2 fragments and prothrombin fragments [147], with lower levels of chemokines MCP-1 and GRO-α than Duffy antigen positive Caucasians (though no changes in circulating leukocyte or pro-/anti-inflammatory cytokine levels are found) [148]. In general, people of African ancestry have lower cytokine and CRP responses to LPS than those of European ancestry [92].

Diurnal variation of should also be considered; secretion of several cytokines in response to LPS is higher at night [149].

Additionally, most IV models deliver LPS as a single bolus, which may not accurately represent prolonged stimuli in critical illness. Similarly, sepsis and ARDS have a range of causes with variable outcomes whereas LPS is a single insult that represents only one of the virulence factors of Gram-negative bacteria. Additionally, models will not account for the modification of the clinical picture caused by interventions such as antibiotics, fluid resuscitation and organ support [150]. Current models also tend to give interventions prior to LPS exposure, which poorly reflects the temporal relationship of illness and therapy. Finally, the magnitude of the inflammatory response that can be induced in human volunteers is obviously restricted by ethical constraints.

For inhaled LPS models, the use of commercial LPS sources could obscure the dose-response relationship, though some more recent models have used CCRE LPS, generally at a dose of 20,000 endotoxin units (EU) (equivalent to 2μg) (see Table 2b). Inhaled LPS models are subject to greater experimental variation than others; different nebulisers produce particles of different sizes [140] and different lung distributions [151] which affects the exposure of lung tissue.

As such, current experimental design models may not fully encapsulate the heterogeneity of relevant patient populations. Refinement of LPS models should ensure they are not overly reductionist and remain clinically relevant. Furthermore, LPS challenge may only model a specific endotype of a condition (e.g. hyper-inflammatory ARDS) which partially explain the failure to translate therapies demonstrating efficacy in LPS models into successful treatments for critical illness.

Reproducibility of response to LPS

Human LPS models are considered to provide a means of generating a controlled inflammatory response in a reproducible manner. Both bronchoscopic and inhaled LPS respiratory challenges have been shown to produce consistent intra-subject cytokine responses to recurrent challenges (regardless of time between exposures for inhaled LPS) [114, 152, 153]. In IV models similar ratios of induced cytokines have been demonstrated in the same subjects when the exposures are separated by at least 5 weeks [69].

A review of the literature demonstrates that across thousands of volunteers, exposure to LPS generates a predictable response, with localised/systemic

neutrophilia and induction of pro- and anti-inflammatory cytokines to broadly similar levels. This is consistent with the personal experience of authors of this review. However, even when models are restricted to phenotypically similar subjects there exists the potential for inter-subject variability. Variable inflammatory responses are an important aspect of human LPS models, as they may help us understand why some patients develop conditions such as ARDS, whilst others, under the same circumstances, do not.

Underlying genetic differences may contribute to this variability, though the relationship is complex; neither the well characterized TLR4 receptor co-segregating mis-sense mutations 'Asp299Gly' and 'Thr399Ile' nor a number of other frequently studied polymorphisms in genes coding for TNF-α, IFN-γ and IL-10 have effect the response to IV LPS [154–156] (though the TLR4 mutations do result in a reduced acute phase protein and leukocyte response after LPS inhalation [157]).

Polymorphisms altering the TNF-α response have perhaps been focused on most. Mutations in the genes coding for MMP-8 (259A/G), resulting in suppressed MMP-8 production) [158], CRP (+1444C/T polymorphism) [159], the β-chain of the fibrinogen molecule (-148C/T polymorphism) [160], result in lower TNF-α levels, whereas mutations in apolipoprotein E alleles cause significantly higher levels of TNF-α to be produced [161].

The cellular and haemostatic responses are also subject to genetic variation; a mutation in the gene coding for E-selectin (561A/C) results in significantly higher F1+2 and d-dimer fragment generation [162], as does the +1444C/T CRP mutation

[159]. People who are homozygous for a 163G/T mutation in the coagulation factor XIII-A gene subunit have a less pronounced neutrophil response and smaller fall in circulating monocyte numbers in response to IV LPS [163], whilst the E-selectin polymorphism results in monocyte counts 20% higher at 24 hours [162].

The response to inhaled LPS also has a genetic component, potentially linked to a deletion polymorphism in an anti-oxidant gene, *glutathione-S-transferase Mu1* (GSTM1), present in 40-50% of the population, resulting in increased circulating platelet and total leukocyte counts, and increased sputum neutrophil counts, in response to inhaled LPS [164].

FUTURE CONSIDERATIONS

LPS models do not replicate critical illness, although some similar physiological alterations are observed [65] and changes in clinical and inflammatory parameters have been shown to be broadly similar in LPS models and sepsis [23, 165]. When considering lung injury, raised levels of chemotactic mediators (such as MCP-1) and newly characterised inflammatory proteins like whey acidic protein four-disulfide-core 12 (WFDC12) have been demonstrated in healthy volunteers challenged with LPS and ARDS [136, 166].

LPS models provide an important link between basic science and clinical trials, offering the ability to generate an inflammatory response from a defined and accessible time point in human biological systems in a safe, reproducible manner [114, 152, 153].

In vivo LPS models allow inflammation and its interaction with other organ systems, such as cardiovascular or neuro-endocrine responses, to be studied in the whole organism. The findings have greater inference than studies from isolated blood components. For example, it has been shown that *ex vivo* cytokine production cannot be used to predict the *in vivo* inflammatory response [167] and that endotoxin tolerance follows different kinetics in *in vivo* models compared to *ex vivo* ones [68].

Furthermore, they are useful for proof of concept studies; negative findings in LPS models should give rise to consideration about whether further investigation is warranted.

Future research may benefit from several adjustments to the LPS models currently in use. Standardisation of the source of LPS, dosing criteria and variables measured to assess response, would help comparisons between studies. Expanding models to include study cohorts comprised of women, older subjects and those with chronic diseases may make observations more generalisable.

LPS tolerance must be taken into account when considering experimental design, particularly in IV challenge. Appropriate washout periods must be used. Repeated LPS doses are most often given in the investigation of tolerance kinetics [26, 69] or during cross-over studies; generally these include a washout period of >5 weeks [38, 41, 90, 168] However, several studies have given repeated doses of IV LPS with washout periods <2 weeks [83, 85] or not specified [169], suggesting observations could be affected by a tolerance. Induction of LPS tolerance can be used to study

the immunosuppressed phenotype of critical illness, with various groups demonstrating it can be reversed with IFN-γ [170] or aspirin [171].

Infusing LPS over several hours may better represent ongoing pathogen exposure. A bolus-followed-by-infusion model resulted in higher cytokine levels and longer duration of pyrexia compared to a bolus of 1ng/kg (although the total amount of LPS delivered was three times greater) [172]. Low doses (0.3ng/kg) produce an earlier and more pronounced inflammatory response when delivered via a bolus compared to an infusion [173]. Several newer studies have used an LPS infusion over 4 hours [174–177]. Further exploration of these models may allow the evaluation of interventions in the setting of ongoing inflammation and immunosuppression that occurs in sepsis, though repeated exposure places more onerous requirements on both volunteers and researchers. Another strategy may be greater use of models that deliver the intervention after LPS challenge, more accurately replicating real life. This may lead to more rapid exclusion of treatments which only work when applied in an antecedent manner. Finally, rapid advances in '-omics' technology will provide opportunities to dissect the human inflammatory response using LPS, and to compare these responses with endotypes derived from observational cohorts.

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COMPETING INTERESTS

The authors have no competing interests to declare.

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FIGURE LEGENDS

Figure 1: A schematic diagram of the lipopolysaccharide molecule.
M = monosaccharide; Ga = galactose; Gl = glucose; H = heptose; KDO = 2-keto-3-deoxyoctonic acid; G = glucosamine; C = carbon.

Figure 2: A summarised, simplified version of LPS signaling in the human body (for a detailed review see refs [19, 20, 22]).

Figure 3: Graphical summary of the effects of intravenous LPS on clinical, haematological, cellular and inflammatory parameters constructed from papers infusing 2ng/kg CCRE LPS to healthy volunteers. To provide an overview of the effects of IV LPS, the average (mean) values of clinically important parameters were calculated from individual papers where data were available for that parameter. Clinical parameters were constructed from yet to be published data from a 2ng/kg IV LPS model within our own lab.
For graphs with two x-axes, the parameters displayed on the graph should be read relative to the axis under which they are listed. For example, those listed on the left hand side of the figure should be read against the left-sided x-axis.
Cellular: neutrophils [28, 51, 71, 178]; macrophages [71, 178, 179]; lymphocytes [28, 71, 178, 179].
Cytokine: TNF-α [8, 24, 28, 51, 71, 72, 75, 178]; IL-6 [8, 24, 28, 43, 51, 72, 178]; IL-8 [8, 24, 43, 72, 178]; IL-10 [8, 43, 51, 72, 178]; TNF-SRI [28, 75].
Haematological: F_{1+2} [40, 43–45, 47, 49]; TAT complexes [43–45, 49]; tPA [47, 49, 75]; sTF [39, 47, 73].

Figure 4: Graphical summary of the effects of respiratory LPS models. Inhaled LPS responses displayed are induced sputum cell counts and induced sputum cytokines. Instilled LPS responses displayed are BAL cell counts, BAL cytokine levels and BAL haematological parameters. To provide an overview of the effects of respiratory LPS models, the average (mean) values of clinically important parameters were calculated from individual papers where data were available for that parameter. To ensure consistency of responses only papers using CCRE endotoxin (20,000 endotoxin units (equivalent to 2μg) for inhaled papers and 4ng/kg for instilled papers) were included.
Induced sputum cellular: neutrophils [102, 122, 152, 180–182]; macrophages [102, 152, 180, 182].
For graphs with two x-axes, the parameters displayed on the graph should be read relative to the axis under which they are listed. For example, those listed on the left hand side of the figure should be read against the left-sided x-axis.
Induced sputum cytokines: IL-8 [152, 180, 182]; IL-6 [180–182]; TNF-α [180, 181].
BAL cellular: neutrophils [109, 111, 113, 114, 118, 183]*;* macrophages [111, 113, 114, 118]; lymphocytes [113, 114].
BAL cytokines: TNF [111, 113, 114, 118, 183]; IL-6 [111, 113, 114, 118, 183]; IL-8 [111, 113, 114, 118, 183]; TNF-SRI [111, 114, 183].
BAL haematological: F_{1+2} fragments [118]; TAT complexes [117–119]; sTF [117–119]; tPA [117, 119].

Table 1a – Summary of source and dose of LPS and subject characteristics in studies using intravenous LPS models

LPS source	Subjects	Dose	References
CCRE[&]	Healthy males and females, aged 18-45*	4ng/kg	[27, 29, 30, 37, 46]
CCRE	Healthy males, aged 18-45*	4ng/kg	[31, 32, 39, 41, 42, 48, 53, 62, 73, 84, 90, 110, 165]
CCRE	Healthy males and females, aged 23-52	1, 2, 3 or 4 ng/kg	[25]
CCRE	Healthy males, aged 22-49	1, 2 or 4ng/kg	[9]
CCRE	Healthy males, age not specified	1, 2 or 4 ng/kg	[63]
CCRE	Healthy males, aged 18-30	3ng/kg	[52]
CCRE	Healthy males and females, aged 18-45*	2ng/kg	[8, 28, 36, 61, 64, 65, 72, 86, 89, 145, 156, 161, 167]
CCRE	Healthy males and females, aged 20-27 and 61-69	2ng/kg	[143, 144]^
CCRE	Healthy males, aged 18-45*	2ng/kg	[26, 38, 40, 43–45, 47, 49, 50, 70, 71, 74, 75, 77–79, 81–83, 85, 91, 146–148, 154, 158–160, 162, 163, 168–170]
CCRE	Healthy males, without age specified	2ng/kg	[51, 68, 178]
CCRE	Healthy subjects, without age or gender specified	2ng/kg	[60, 94, 179]
CCRE	Healthy males, aged 18-28	0.5, 1 and 2ng/kg sequentially	[24]
CCRE	Healthy males, aged 18-28	0.5, 1 or 2ng/kg	[87]
CCRE	Healthy males and females, aged 18-45	1ng/kg	[92]
CCRE	Healthy males, aged 25-40	1ng/kg	[69]
CCRE	Healthy males and females, aged 18-45	0.6ng/kg	[93]
CCRE	Healthy males, aged 18-45*	0.5ng/kg/hr for 4 hours	[23, 174–176]
CCRE	Healthy males and females, aged 22-41	0.4ng/kg	[142]
CCRE	Healthy males, aged 19-31	0.3ng/kg	[149]
CCRE	Healthy males, aged 18-45	0.1ng/kg	[155]
CCRE	Healthy males, aged	Bolus	[171, 173, 177]

	18-35	1ng/kg then 3 hour infusion of 1ng/kg/hr	

*to reduce table size multiple age ranges from different studies collapsed into single category
[&]Clinical Centre Reference Endotoxin
[^][143] does not specify source of LPS, however previous papers by author have used CCRE.

Table 1b – Summary of source and dose of LPS and subject characteristics in studies using pulmonary LPS models

Inhaled nebulized LPS			
LPS source	Subjects	Dose	References
Sigma	Healthy males and females and asthmatic males and females, aged 18-45[^]	100μg	[99]
Difco	Healthy males and females, age not specified	100μg	[101]
Sigma	Healthy males, aged 18-45	100μg	[116, 125]
Sigma	Healthy males and females, aged 18-40	60μg	[127]
Sigma	Healthy males, aged 18-40	60μg	[130–132, 139]
Sigma	Healthy males, atopic males and asthmatic males, aged 18-45[^], male	60μg	[6]
Sigma	Healthy controls, smokers and pig farmers, males and females, aged 22-61	53.4μg	[97]
Sigma	Healthy males and females, aged 18-60	50μg	[103–106, 136]
Sigma	Healthy males, aged 18-45	50μg	[115]
Sigma	Healthy males and females, aged 18-45	0.5, 5 and 50μg sequentially	[96]
Sigma	Healthy subjects, without age or gender specified	5 and 50μg	[98]
Sigma	Healthy males and females, aged 26-39	15 or 50μg	[95]
Sigma	Healthy males and females, aged 20-30	40μg	[124]
Sigma	Healthy non-smokers and smokers, aged 31-43, gender not specified	30μg	[7]
Sigma	Healthy males and females, aged 18-55*	20μg	[120, 121, 140, 157]
Sigma	Healthy males and females, aged 18-59*	0.5, 1.0, 2.0, 3.0, 5.0, 10 and 20μg sequentially	[100, 126]
CCRE	Healthy males and females and non-smokers and smokers, aged 19-48	20000 EU[$]	[102, 122]
CCRE	Healthy males and females, aged 18-55*	20000 EU	[152, 153, 180, 182]
CCRE	Healthy subjects, with age or gender specified	20000 EU	[164]
CCRE	Healthy males and females and atopic asthmatics	20000 EU	[181]
Sigma	Healthy males and females, aged 28-34	15μg	[123]

CCRE[&]	Healthy males and females, aged 18-50	0, 2500, 5000 and 10000 EU[$] sequentially	[129]
Bronchoscopically instilled LPS			
CCRE	Healthy males and females, aged 18-45*	4ng/kg	[109, 118, 119, 134, 183]
CCRE	Healthy males, aged 18-50*	4ng/kg	[110–114, 117, 141]
CCRE[%]	Healthy subjects, age and gender not specified	4ng/kg	[133][%]
CCRE	Healthy males and females, aged 26-31	1, 2 or 4ng/kg	[108]
CCRE	Healthy males and females, age not specified	1, 2 or 4ng/kg	[107]

*to reduce table size multiple age ranges from different studies collapsed into single category
[^]mean/median age given but range not specified
[$]endotoxin units
[%]Refers to same protocol as [108] but doesn't specifically state source or dose of LPS used
[&]Clinical Centre Reference Endotoxin

Table 1c – Summary of source and dose of LPS and subject characteristics in studies using nasal and intradermal LPS model

Nasal LPS challenge			
LPS source	Subjects	Dose	References
Sigma	Healthy subjects, age and gender not specified	100μg	[14]
Invivogen	Healthy males and females, aged 21-57	1, 10, 30 and 100μg	[15]
Sigma	Healthy males and females, aged 26-43	0, 10 and 40μg	[13]
Intradermal LPS challenge			
LPS source	Subjects	Dose	References
CCRE	Healthy males and females, aged 18-50	15ng	[16]

Table 2a. Summary of biochemical/cytokines changes from intravenous LPS models

Intravenous LPS					
Raised over time course					
1 hour	2 hours	4 hours	6 hours	12 hours	24 hours
Pro-inflammatory cytokines					
TNF-α	TNF-α	TNF-α			
IL-6	IL-6	IL-6			
IL-8	IL-8	IL-8	IL-8		

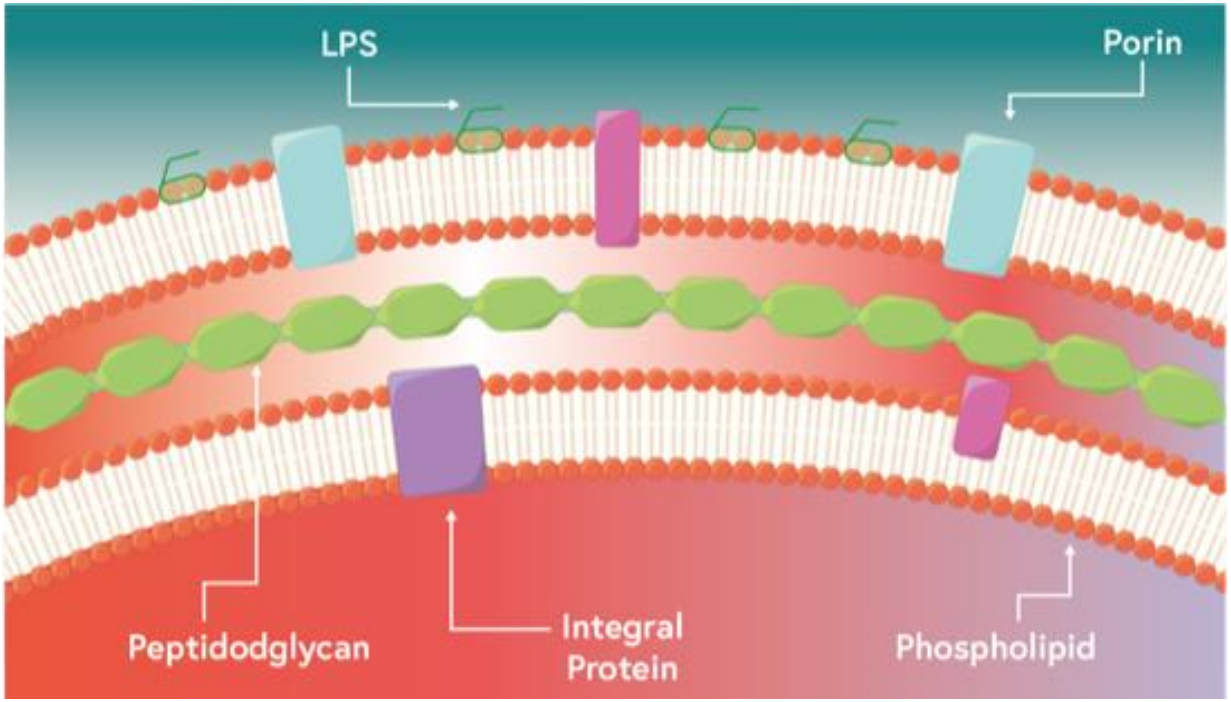
IFN-γ	IFN-γ				
Anti-inflammatory cytokines					
IL-10	IL-10	IL-10	IL-10		
TNF-R	TNF-R	TNF-R	TNF-R		
	IL-1Ra	IL-1Ra	IL-1Ra	IL-1Ra	
Chemoattracts and other molecules					
MIP-1α	MIP-1α				
GRO-α	GRO-α				
	MCP-1	MCP-1	MCP-1		
	MIG	MIG	MIG	MIG	MIG
	IP-10	IP-10	IP-10		
	Cortisol	Cortisol	Cortisol		
				CRP	CRP
Markers of cellular activation					
Granzyme A	Granzyme A				
Granzyme B	Granzyme B	Granzyme B			
	Lactoferrin	Lactoferrin	Lactoferrin	Lactoferrin	
	HNE	HNE	HNE	HNE	HNE

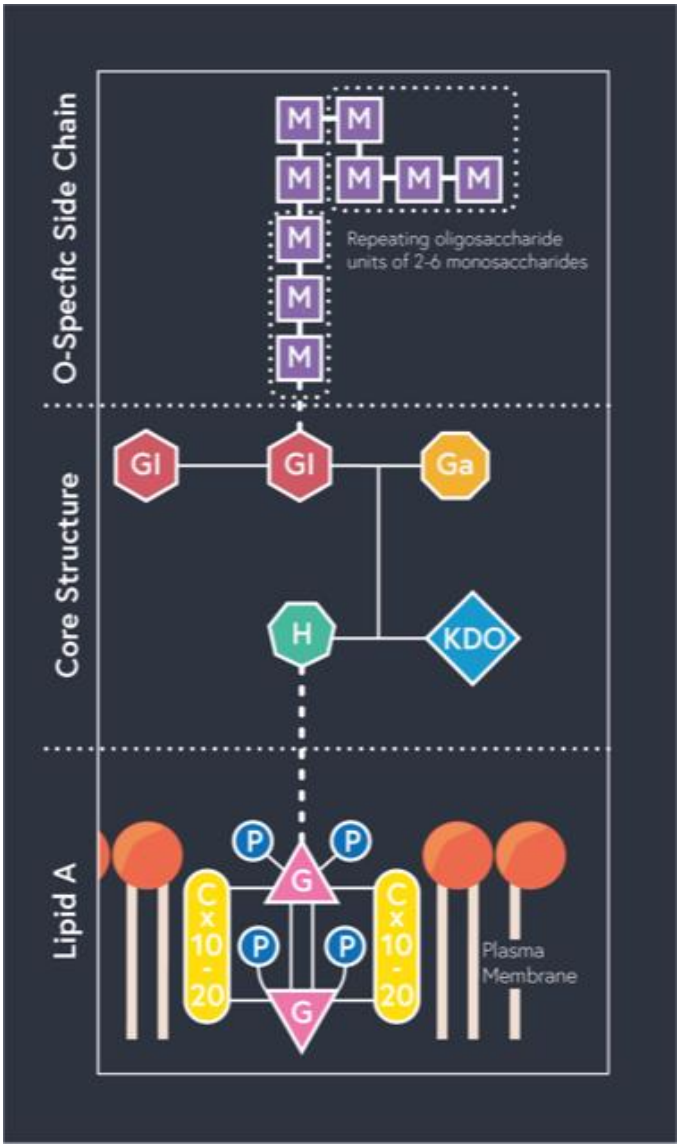
Table 2b. Summary of biochemical/cytokine changes from inhaled/instilled LPS models

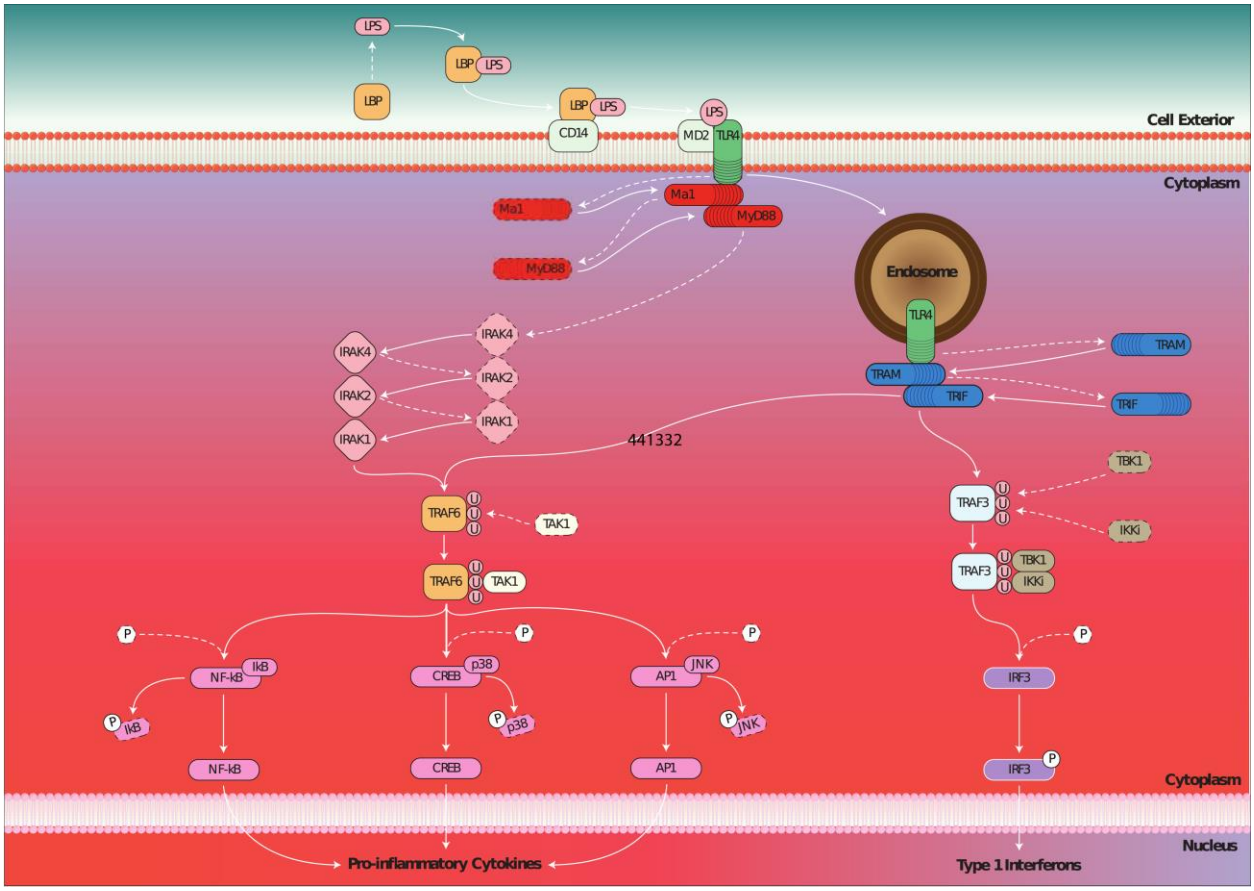
Inhaled LPS				**Bronchoscopically instilled LPS**			
Raised over time-course							
90 minutes	4-6 hours	24 hours	No change	Raised at 6 hours	Raised at 24 hours	Raised at 48 hours	No change
Sputum							
	TNF-α			-	-	-	
	IL-6			-	-	-	
	IL-8	IL-8		-	-	-	
	MIP-1β			-	-	-	
BALF							
Pro-inflammatory cytokines							
TNF-α	TNF-α	TNF-α		TNF-α	TNF-α		
IL-1β	IL-1β			IL-1β	IL-1β		
IL-8	IL-8	IL-8		IL-8			
	IL-6			IL-6	IL-6		
				G-CSF	G-CSF		
Anti-inflammatory products							
				IL-1Ra			
				TNF-R	TNF-R	TNF-R	
Neutrophil derived products							
	MPO			MPO	MPO	MPO	
	HNE			HNE			
				Lactoferrin	Lactoferrin	Lactoferrin	
					MMP-8		
					β-2-def		
Chemoattractants							
	MIP-1β			MIP-1α			
	ENA-78			ENA-78			
	MCP-1			MCP-1			
				IP-10			
				GRO-α	GRO-α	GRO-α	

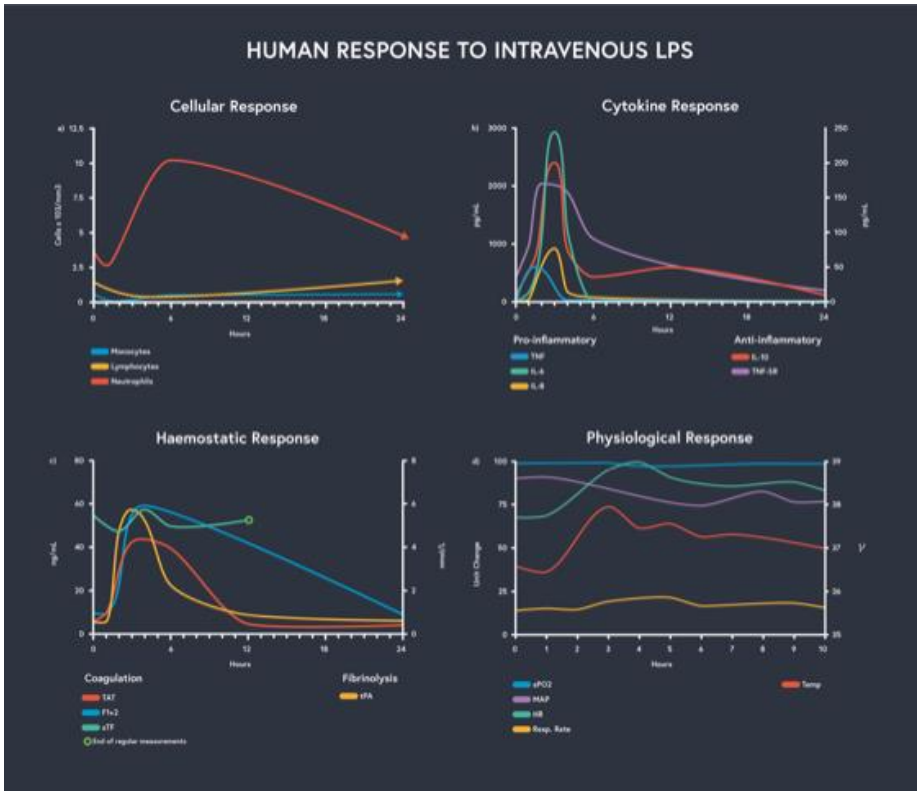
Markers of endothelial damage/activation							
	sTM						
Systemic							
Pro-inflammatory cytokines							
				Raised 2-24 hours			
	IL-6			*IL-6*			
	IL-8						
				G-CSF			
			TNF-α				TNF-α
Anti-inflammatory products							
				IL-1Ra			
			IL-10				
Neutrophil derived products							
		MPO					
	HNE						
Chemoattracts and other molecules							
			IL-1β				
			MCP-1				
		CRP			CRP		
		α_1-AT					
Markers of endothelial damage/activation							
	E-selectin						

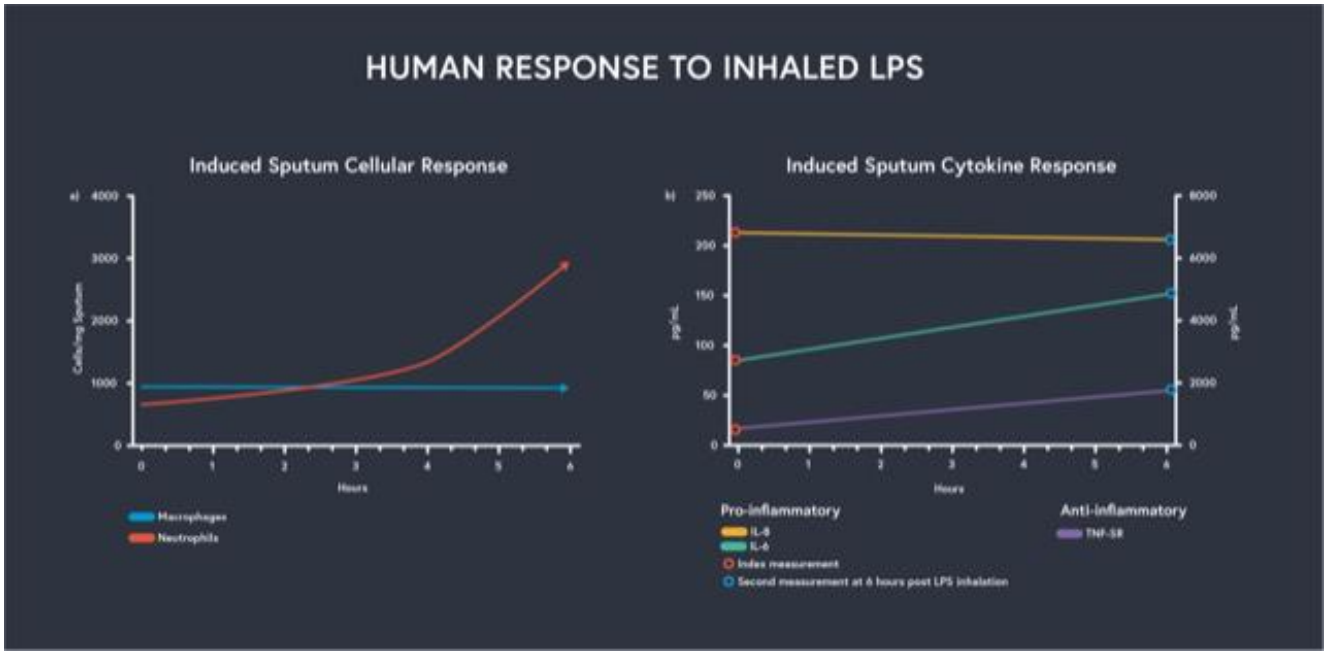
Tabular summary of changes in biochemical/cytokine parameters from all papers cited in this review. Obtained from the following references:
Intravenous: TNF-α [9, 27, 28, 71]; IFN-γ [72]; IL-6 [9, 24, 27, 28, 51]; IL-8 [9, 24, 27, 51]; granzyme A and B [73], HNE [26, 42]; lactoferrin [25, 40]; MIP-1α [27]; GRO-α [27, 28]; MCP-1 [42]; MIG, IP-10 [73]; IL-10 [27, 42, 43, 51, 72]; IL-1Ra [25, 27, 28, 42, 51]; TNF-R [25, 27, 42, 75]; cortisol [8, 9, 27, 71]; CRP [9, 24, 72].
Inhaled sputum: IL-6, IL-8, TNF-α [6, 95, 97, 123]; MIP-1β [123].
Inhaled BALF: TNF-α [7, 105, 125, 126]**;** IL-1β [7, 125, 126]; IL-6 [105, 125]; MPO, HNE [125, 132]; MIP-1β, (ENA)-78, MCP-1 [125, 136], sTM [116]
Inhaled systemic: IL-6, IL-8, TNF-α, IL-1β, IL-10, MCP-1 [122, 123, 132]; HNE [132]; MPO [124]; CRP, α_1-antitrypsin [104, 120], E-selectin [96, 104, 140]
Instilled BALF: TNF-α, IL-1β, IL-6, IL-8, G-CSF [108, 111, 113, 133]; IL-1Ra and TNFR [108]; ENA-78, lactoferrin, MPO, HNE, MIP, GRO-α, IP-10, MCP-1 [108, 111]; MMP-8, β-defensin-2 [133].
Instilled systemic: G-CSF, IL-6, IL-1Ra, CRP [108, 110]**.**

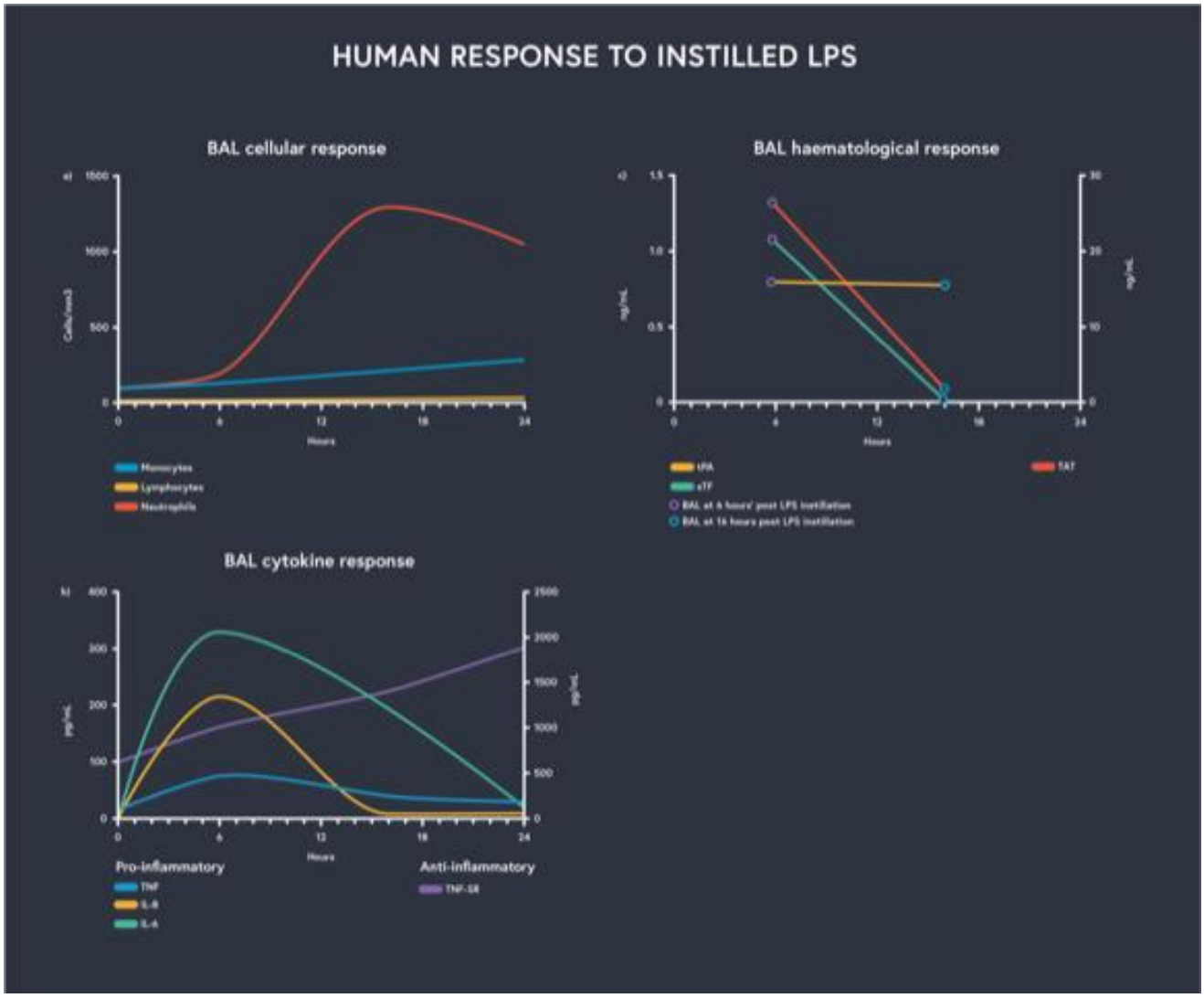

LPS
Porin
Peptidodglycan
Integral
Protein
Phospholipid


O-Specfic Side Chain
M
M
M
M
M
M
M
M
M
Repeating oligosaccharide
units of 2-6 monosaccharides
Core Structure
Gl
Gl
Ga
H
KDO
Lipid A
P
P
G
P
P
G
C
x
10
-
20
C
x
10
-
20
Plasma
Membrane


LPS
LBP
LBP LPS
LBP LPS
CD14
LPS
MD2
TLR4
Cell Exterior
Cytoplasm
Ma1
Ma1
MyD88
MyD88
Endosome
TLR4
TRAM
TRAM
TRIF
TRIF
IRAK4
IRAK4
IRAK2
IRAK2
IRAK1
IRAK1
441332
TBK1
TRAF6
TAK1
TRAF3
IKKi
TRAF6
TAK1
TRAF3
TBK1
IKKi
P
P
P
P
NF-kB
IkB
CREB
p38
AP1
JNK
IRF3
P
IkB
P
p38
P
JNK
NF-kB
CREB
AP1
IRF3
P
Cytoplasm
Nucleus
Pro-inflammatory Cytokines
Type 1 Interferons


HUMAN RESPONSE TO INTRAVENOUS LPS
Cellular Response
Hours
Monocytes
Lymphocytes
Neutrophils
Cytokine Response
Hours
Pro-inflammatory
TNF
IL-6
IL-8
Anti-inflammatory
IL-10
TNF-SR
Haemostatic Response
Hours
Coagulation
TAT
F1+2
sTF
End of regular measurements
Fibrinolysis
tPA
Physiological Response
Hours
ePO2
MAP
HR
Resp. Rate
Temp


HUMAN RESPONSE TO INHALED LPS
Induced Sputum Cellular Response
a)
4000
3000
2000
1000
0
Cells/mg Sputum
0
1
2
3
4
5
6
Hours
Macrophages
Neutrophils
Induced Sputum Cytokine Response
b)
250
200
150
100
50
0
pg/mL
8000
6000
4000
2000
0
pg/mL
0
1
2
3
4
5
6
Hours
Pro-inflammatory
IL-8
IL-6
Index measurement
Second measurement at 6 hours post LPS inhalation
Anti-inflammatory
TNF-SR


HUMAN RESPONSE TO INSTILLED LPS
BAL cellular response
Cells/mm3
Hours
Monocytes
Lymphocytes
Neutrophils
BAL haematological response
ng/mL
Hours
tPA
TAT
sTF
BAL cytokine response
pg/mL
Hours
Pro-inflammatory
TNF
IL-8
IL-6
Anti-inflammatory
TNF-SR