



HAL
open science

The dangerous liaisons in innate immunity involving recombinant proteins and endotoxins: Examples from the literature and the *Leptospira* field

Delphine Bonhomme, Jean-Marc Cavaillon, Catherine Werts

► To cite this version:

Delphine Bonhomme, Jean-Marc Cavaillon, Catherine Werts. The dangerous liaisons in innate immunity involving recombinant proteins and endotoxins: Examples from the literature and the *Leptospira* field. *Journal of Biological Chemistry*, 2024, 300 (1), pp.105506. 10.1016/j.jbc.2023.105506 . pasteur-04600357

HAL Id: pasteur-04600357

<https://pasteur.hal.science/pasteur-04600357>

Submitted on 4 Jun 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

The dangerous liaisons in innate immunity involving recombinant proteins and endotoxins: Examples from the literature and the *Leptospira* field

Received for publication, July 24, 2023, and in revised form, November 15, 2023. Published, Papers in Press, November 27, 2023,

<https://doi.org/10.1016/j.jbc.2023.105506>

Delphine Bonhomme¹, Jean-Marc Cavaillon², and Catherine Werts^{1,*} 

From the ¹Institut Pasteur, Université Cité Paris, CNRS UMR6047, INSERM U1306, Unité de Biologie et Génétique de la Paroi Bactérienne, Paris, France; ²Institut Pasteur, Paris, France

Reviewed by members of the JBC Editorial Board. Edited by Clare E. Bryant

Endotoxins, also known as lipopolysaccharides (LPS), are essential components of cell walls of diderm bacteria such as *Escherichia coli*. LPS are microbe-associated molecular patterns that can activate pattern recognition receptors. While trying to investigate the interactions between proteins and host innate immunity, some studies using recombinant proteins expressed in *E. coli* reported interaction and activation of immune cells. Here, we set out to provide information on endotoxins that are highly toxic to humans and bind to numerous molecules, including recombinant proteins. We begin by outlining the history of the discovery of endotoxins, their receptors and the associated signaling pathways that confer extreme sensitivity to immune cells, acting alone or in synergy with other microbe-associated molecular patterns. We list the various places where endotoxins have been found. Additionally, we warn against the risk of data misinterpretation due to endotoxin contamination in recombinant proteins, which is difficult to estimate with the *Limulus* amoebocyte lysate assay, and cannot be completely neutralized (e.g., treatment with polymyxin B or heating). We further illustrate our point with examples of recombinant heat-shock proteins and viral proteins from severe acute respiratory syndrome coronavirus 2, dengue and HIV, for which endotoxin contamination has eventually been shown to be responsible for the inflammatory roles previously ascribed. We also critically appraised studies on recombinant *Leptospira* proteins regarding their putative inflammatory roles. Finally, to avoid these issues, we propose alternatives to express recombinant proteins in nonmicrobial systems. Microbiologists wishing to undertake innate immunity studies with their favorite pathogens should be aware of these difficulties.

In 1989, the exact nature of the immune system was reexamined by Charles Janeway. He proposed that the self and nonself model had reached its end and argued that the innate immune system was the true gatekeeper (1). He also argued that the innate immune system uses ancient pattern recognition receptors (PRRs) to recognize a pathogen by its

unchanging characteristics, the pathogen-associated molecular patterns or the microbial-associated molecular patterns (MAMPs). As a result, there was no longer any reason to consider that there was a specific and a nonspecific immunity. Polly Matzinger, on her side, considered that the immune system reacts to endogenous danger signals generated by cells and tissues in response to any sterile or microbial insult, the so-called “damage-associated molecular patterns” (2). Since the innate immune system has regained major interest and many efforts have been devoted to deciphering it. Its study has focused on the contribution of immune cells such as macrophages whose role was reported in the XIXth century by Elie Metchnikoff (3), and dendritic cells, identified more recently by Ralph Steinman (4). These cells are responsible for detecting MAMPs and damage-associated molecular patterns and initiating the immune response. They are therefore highly sensitive to these signals. Among those, Gram-negative bacterial endotoxins, or lipopolysaccharides (LPS) are the most potent agonist. Unfortunately, LPS can contaminate many preparations of molecules whose role in innate immunity has been under investigations. In this review, we propose to examine why false results have been published due to endotoxin contamination and offer some possible avenues to avoid perpetuating these errors.

Historical background and current problems

From the discovery of endotoxins to the establishment of their biological activities

The word “toxin” has been first used in 1888 by Ludwig Brieger (5), a German physician working in Berlin with Robert Koch. In 1892, Richard Pfeiffer, a German physician and bacteriologist, proposed that toxic properties of bacteria were due to a factor released by lysed bacteria, and coined the term “endotoxin”. The link between this bacterial product and its capacity to induce fever in mammals was shown by Nikolai Gamaleïa, and Sir Marc Armand Ruffer (6–9). In 1894, Eugenio Centanni, an Italian pathologist, recognized the intimate relationship between the pyrogenic and the toxic properties of the poison, which he found to be chemically inseparable, and he named his material “pyrotoxina”. Ironically, “pyrexin”, the first endogenous pyrogenic factor claimed

* For correspondence: Catherine Werts, cwerts@pasteur.fr.

to have been isolated by Valy Menkin in 1944 (6) was finally recognized to be contaminated by endotoxin (7). It was in 1953, that interleukin-1 (IL-1) was recognized to be the endogenous pyrogen (8).

In the 1970s, LPS was reported to be a very potent inducer of inflammatory cytokines (9), those being responsible for the endotoxin shock. Indeed, minute amounts of endotoxins can trigger the release of cytokines by mammalian monocytes and macrophages. Synergy between LPS and other MAMPs such as muramyl dipeptide (10) was shown long before the discovery of their respective PRRs. The identification of toll-like receptor 4 (TLR4) as the endotoxin receptor was achieved by Bruce Beutler, who was awarded the 2011 Nobel Prize for this discovery. It resulted from the analysis of peculiar mice, C3H/HeJ, which are insensitive to LPS because of a mutation in the *tlr4* gene (11). In contrast to humans and rabbits, mice and rats are highly resistant to endotoxin (12). Despite this major difference, LPS has been widely used in murine models to mimic sepsis, but no new effective drug has emerged that has clearly improved patient outcomes (13). Indeed, while large amounts of LPS do induce a major inflammation in mice, it cannot be used as a model of sepsis (14). New methods, such as single-cell RNA-sequencing; single cell analysis and next-generation sequencing, have allowed to further decipher the immune and cellular responses of murine and human cells to LPS at the molecular level (15–17).

Structure and variability of LPSs

The first biochemical characterization of endotoxin was achieved in 1935 by Lydia Mesrobian and André Boivin. They called it “antigène glucido-lipidique,” before it was renamed lipopolysaccharide (18). In 1952, Otto Westphal described the most widely used method to prepare LPS from

Gram-negative bacteria by a hot aqueous phenol extraction (19). Endotoxins make up 75% of the outer membrane, and there are an estimated 4 million LPS molecules *per* bacterial cell. More importantly, despite the use of a common acronym for this bacterial compound, there is a wide chemical diversity of LPSs depending on the bacterial origin (20–22). This extreme heterogeneity underlines the diverse biological properties reported depending on the nature of the LPSs (23). The complete structure is made up of external repetitive units of oligosaccharides defining the O-antigen, a polysaccharidic core, and a lipidic moiety bound to a dimer of glucosamine, called lipid A (Fig. 1). The complete structure can be found in *Enterobacteriaceae*, *Pseudomonas*, *Leptospira* and *Vibrio*. A great diversity of oligosaccharides has been identified and some rare sugars, specific to the microbial world have been found (e.g., tyvelose) (24). The number of repetitive units can vary and fluctuates within a given preparation. The O-antigen can be absent as it is the case for *Neisseria meningitidis*, *Bordetella pertussis*, *Acinetobacter*, and *Bacteroides fragilis*. The canonical full structure is responsible for the “smooth” phenotype of colonies, whereas LPS devoid of O antigen define a “rough” phenotype. The core is made of variable sugar residues, but the inner core is rather conserved, made up with heptose and 2-keto-3-deoxyoctonate which is linked to the lipid A. The outer core can be absent as it is the case for *Chlamydia* and *Rhodospseudomonas*. The lipid A is made of a β 1,6-linked glucosamine dimer backbone, phosphate groups at the 1 and 4' positions of the backbone and fatty acids bound to the dimer of glucosamine. Regarding the lipid A, the variability concerns the number of fatty acids (from 4 to 7), the length of the acyl chain (from 10 to 18 carbons) and the addition of various substitutes to the dimer of glucosamine (phosphate group, ethanolamine, glucosamine and so on). The biological activity of LPS is mainly dependent on the lipid A and the

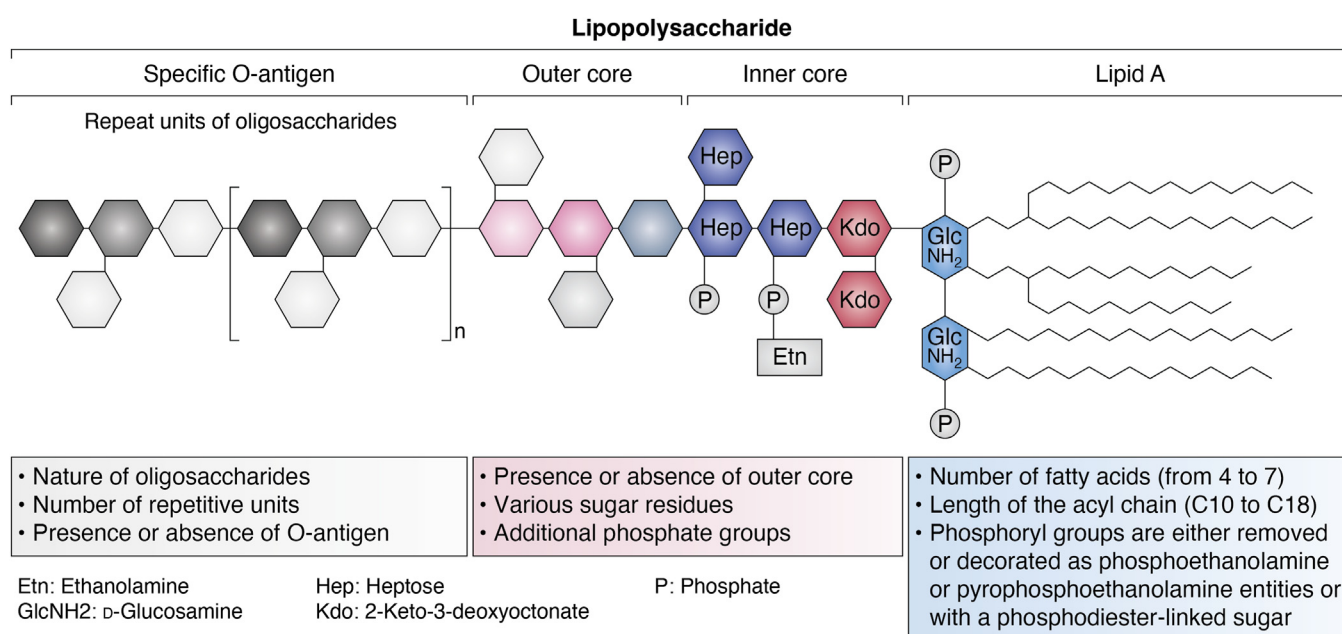


Figure 1. Structure of lipopolysaccharides (LPS). Schematic representation of the structural variability of bacterial lipopolysaccharides, composed of the lipid A (lipidic anchor), the inner and outer cores, and the O-antigen with various sugar unit repeats. LPS, lipopolysaccharides.

inner core, whereas the O antigen is immunogenic and defines the serovars or serogroups. Penta-acylated lipid A are poorly active as compared to hexa- or hepta-acylated LPS as observed for example when comparing *B. pertussis* LPS versus *N. meningitidis* LPS (25). In *Leptospira interrogans*, the lack of phosphate in position 4' of the disaccharide, the methylation of the phosphate group in position 1, and four amide chains constitute the most striking features of this unusual LPS, whose weak activity differs from the one from *Escherichia coli* (26, 27), while the great diversity of O antigens defines more than 300 serovars (see [Examples from the leptospiral field section](#)).

Different parameters can influence the LPS biochemical structure. In particular, bacterial culture at different temperature (e.g., 27 °C versus 37 °C) can modulate the number of fatty acids, as seen with *Yersinia pestis* (28). Similarly, growth of bacteria in biofilms results in modifications of LPS structures when compared to planktonic cultures, as demonstrated with *Pseudomonas aeruginosa* (29). Most interestingly, important differences in lipid A structures have been reported when comparing *P. aeruginosa* LPS derived from clinical isolates versus laboratory strains, which were accompanied with major differences in terms of human (but not murine) macrophages activation (30). Finally, another source of heterogeneity lies in the fact that a preparation of LPS or lipid A from a given bacterial origin is not homogeneous and contains different forms as revealed by negative-ion MALDI mass-spectra analysis (31). Furthermore, those forms may vary from batch to batch. Thus, it is important to speak about LPSs,

not as a global entity but as a huge family of similar but not identical molecules.

Natural receptors: endotoxins have high protein binding potential

Many proteins naturally binding to endotoxins have been identified in plasma, saliva, lungs and in granules of neutrophils (Fig. 2). Some have been reported to be acute phase proteins (LPS-binding protein, [LBP]; soluble cluster of differentiation 14 [sCD14]) and to further enhance cell activation by LPS (32, 33). LBP has been shown to behave as a shuttle molecule, catalyzing its interaction with cluster of differentiation (CD)14. LBP is a 60 kDa glycoprotein produced by hepatocytes. Its normal concentration in serum is 3 µg/ml and can reach 200 µg/ml in acute-phase serum. LBP can either shuttle the LPS to plasma high-density lipoprotein for its neutralization or to CD14 for initiation of activation (34). Membrane CD14 is a glycosylphosphatidylinositol-anchored protein with leucin-rich repeats like those found in the extracellular domain of TLR molecules. Apart from being an acute-phase protein, sCD14 can also be released by monocytes/macrophages. sCD14 allows CD14-negative cells, which express TLR4, such as the endothelial cells, to respond to LPS. Interestingly, both anti-LBP and anti-CD14 antibodies protect mice against a lethal challenge with LPS, but these antibodies are deleterious when used during a Gram-negative infection (35). In contrast, other binding-proteins inhibit LPS activating properties (e.g., cationic antimicrobial peptide CAP 18 and

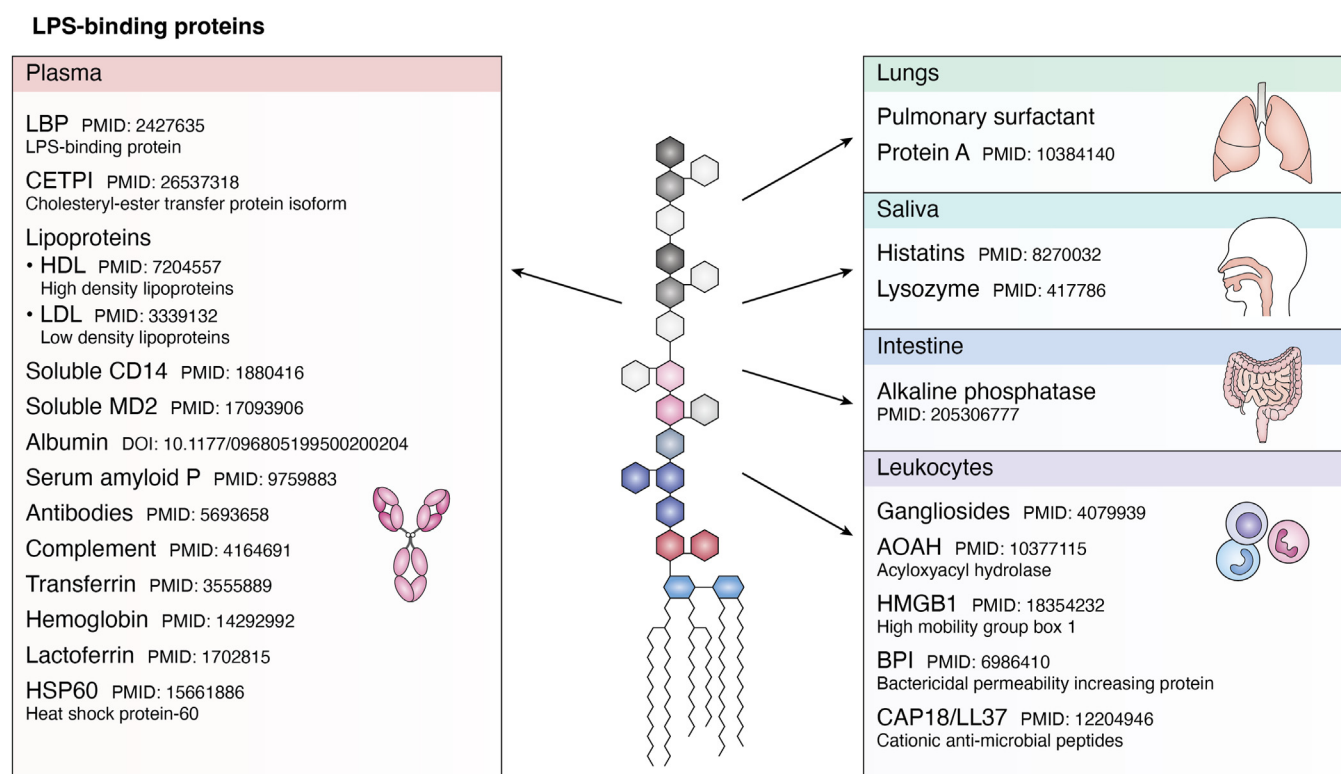


Figure 2. Proteins naturally binding to LPS are numerous. List of proteins naturally binding to LPS and found in different compartments of the human body. LPS, lipopolysaccharides.

CAP 37; bactericidal/permeability increasing protein (BPI)). As reflected in the different LPS structures, their inhibitory potency varies from LPS to LPS (36). BPI shares 44% homology with LBP, mainly present in granules of neutrophils, and it can be found in low amounts in human plasma (0.2–2 ng/ml). In abscess fluids, its concentration exceeds that of LBP (37). BPI is protective against bacterial infection, but its protective efficiency depends on the nature of the bacteria (38). A recombinant fraction of BPI was shown to significantly reduce the neuro-injury and the need for amputation in patients with severe meningococcal sepsis (39). Plasma lipoproteins prevent LPS to interact with its receptor, hence limiting its capacity to activate cytokine release (40). Robert Munford's team has described that an enzyme present in macrophages and neutrophils, the acyloxyacyl hydrolase, removes secondary acyl chains from the lipid A, thus detoxifying the endotoxin molecule (41). Among leukocytes compounds, cell-membrane gangliosides also interact with LPS and prevent their binding to macrophages (42). Among the plasma compounds able to bind endotoxin, albumin is the most abundant one. Lipid A binds albumin with a stoichiometry of 2:1 (43). Albumin has been reported to be a facilitator for the binding of LPS to LBP and CD14 (44). The binding of LPS to albumin was recognized as a nonspecific one (45). In the early efforts to define the LPS receptor, a 70 kDa molecule was identified and finally recognized to be albumin (46). However, a specific receptor was identified in accordance with the definition of a receptor (*i.e.*, saturability, reversibility, and specificity) (47). It was later identified as the CD14 molecule (48, 49). TLR2 (50, 51) and TLR4 (11) were subsequently identified as the receptors that contribute to cell signaling. In fact, it was further proposed that TLR2 was the receptor for rare endotoxins. However, its involvement was due to contaminants present in LPS preparations (52), or to lipoproteins tightly associated with the leptospiral LPS (53, 54). Finally, it was shown that the myeloid differentiation factor 2 (MD2) molecule associated to the extracellular domain of TLR4 was the effective receptor of LPS within the TLR4/MD2 complex (55).

In vivo detection of endotoxins

Circulating endotoxin can be detected in the blood of sepsis patients. The first demonstration was made in 1963 using a rabbit skin test associating subcutaneous injection of epinephrine (56). The possible presence of endotoxin was then confirmed with the Limulus assay in 1970 (57). The highest levels were associated with the poorest outcome (58). In patients with meningococcal infection, plasma levels of LPS were shown to correlate with circulating chemokines (59), and bacterial DNA (60). Of interest, its presence was also found in patients with Gram-positive bacterial or fungal infection, suggesting that part of its origin can derive from gut translocation (58). Indeed, as an illustration of endotoxin translocation from the gut, circulating endotoxins have been found in many clinical settings including abdominal aortic surgery (61), resuscitated cardiac arrest (62), hemorrhagic shock (63), severe dengue infection (64) (see [Relevant examples from the](#)

[literature](#) section), and many others (65). The removal of endotoxin in sepsis patients has been advocated despite some potential bias (66). In a multicenter, randomized, clinical trial that included 450 adults with septic shock and high circulating endotoxin activity, polymyxin B (PMB) hemoperfusion compared with sham hemoperfusion did not significantly decrease 28-days mortality (67). A post hoc analysis identified patients with abnormal coagulation and hyperlactatemia who may benefit from PMB hemadsorption (68).

Endotoxin contaminations are diverse and ubiquitous

As illustrated earlier with the attempt to isolate the endogenous pyrogen (69, 70), contaminations by endotoxin of numerous preparations have jeopardized many studies investigating the role of these compounds in innate immunity. As summarized in [Figure 3A](#), there is a long list of products of which properties have been associated with endotoxin contaminations. Humans can be exposed to endotoxins present in natural environment, not only in farms and industries linked to agriculture (*e.g.*, textile) but also when exposed to dust at home or in offices. The inhalation of contaminated air can lead to various occupational diseases of which the main symptoms are fever, coughing, dyspnea, general malaise, and an alteration of lung function. The contamination can also be present on solid supports (*e.g.*, wound dressing, surgical gloves and so on) as well as in liquid media (*e.g.*, hemodialysis fluids, contact lenses solutions and so on) in contact with the human body.

In *in vitro* experiments ([Fig. 3B](#)), minute amounts of contaminating LPSs can trigger cells either by themselves or in synergy with other microbial compounds (see [Issues about endotoxin contaminations in recombinant proteins](#) section). For numerous molecules initially considered displaying properties of cell activation, it was finally demonstrated that their reported bioactivity was an artifact consecutive to endotoxin contamination (see [Relevant examples from the literature](#) section). The purification of a given molecule, being either a protein, a sugar, or a nucleic acid, can end to a contaminated product because of the use of contaminated water for the preparation of buffers, the use of contaminated supports such as those used in chromatography or the use of contaminated enzymes or antibodies. Of course, the preparation of recombinant proteins cloned in *E. coli* further adds a chance to end with a contaminated product (see [Relevant examples from the literature](#) section). Regarding the culture media, the greatest caution must be taken for the choice of the medium itself and the additives. For example, the endotoxin contamination of fetal calf serum can vary from 0.008 to 10 ng/ml (71). It should be noted that endotoxin decontamination of a glass Erlenmeyer used for *E. coli* cultures requires heating in a dry oven at 250 °C for 4 h. Therefore, using disposable plastic flasks, tubes, and pipettes is almost mandatory when studying innate immunity. In the future, novel washing technology, already commercially available, will allow the general recycling and reuse of plastic pipette tips. This ecofriendly approach, recommended by the ethic committee of CNRS (the French national scientific research agency), may constitute an additional

A Endotoxin contaminations *in vivo*

Air and environment Poultry housing dust PMID: 22029777 Swine confinements PMID: 23293050 Cotton industry PMID: 17341727 Rice production PMID: 6492187 Agricultural industries PMID: 16395461 Livestock farming PMID: 24280684 Cheese factories PMID: 24812257 Home and offices PMID: 34738178 Cockroach feces PMID: 28106812 Sewage treatment plants PMID: 34678984 Waste water treatment plants PMID: 7992797 Hay dust PMID: 1925440 Humidifiers PMID: 33251102	Natural products Allergens PMID: 1702943 Medicinal herbs PMID: 33132322 Raw or farm milk PMID: 2745822 Infant formula milk powder PMID: 33922125	Drugs Antibiotics PMID: 7380997 Bacteriophage therapy PMID: 15213806 Radiopharmaceutical preparations PMID: 23696179
Water Drinking water PMID: 12224557 Hemodialysis fluid PMID: 25641063 Marine bath water PMID: 24642437	Medical devices Surgical gloves PMID: 1976681 Surgical instrument PMID: 16928962 Microelectrode PMID: 24778808 Wound dressings PMID: 12808594 Titanium implant PMID: 12500875 Prosthetic material PMID: 11922375 Sterile catheters PMID: 7380997 Intravenous solution PMID: 16807850	Vaccines BCG PMID: 13641561 Influenza virus PMID: 893659 Mumps vaccine PMID: 321714 Tetanus toxoid PMID: 321714 Veterinary vaccines PMID: 29935930
	Other media Contact lenses and solutions PMID: 1463420 Radiographic contrast media PMID: 552831	Others Feed additives for livestock PMID: 26768246 Animal diet PMID: 28052076 Electronic cigarette PMID: 31017484 Cigarette smoke PMID: 17852769

B Endotoxin contaminations *in vitro*

Culture medium Culture media PMID: 699956 Fetal calf serum PMID: 9439764 Collagen PMID: 969286 Gelatin PMID: 36476713 Ficoll PMID: 9930943 Medium for cell isolation PMID: 10458490 Culture medium for human <i>in vitro</i> fertilization PMID: 3732534	Mitogens Pokeweed mitogen PMID: 16522770 Concanavalin A PMID: 1151067 Lipoteichoic acid PMID: 11159964 Phytohemagglutinin PMID: 321714 PPD of the tubercle bacillus PMID: 321714	Purified proteins Ovalbumin PMID: 7039979 Allergens PMID: 18771487 Bovine serum albumin PMID: 5924502 Human albumin PMID: 4584590 Hemoglobin PMID: 8041288 β -lactoglobulin PMID: 14657886 Human gamma globulin PMID: 4100051 Antibodies PMID: 9407503 Human placental G-CSF PMID: 6985122 Apolipoproteins A-I PMID: 25778625 Alpha-lactalbumin PMID: 20600530 Monoclonal antibodies PMID: 3112017 Tetanus toxoid PMID: 321714
Water Distilled water PMID: 36554408 Osmosis water PMID: 36554408	Nucleic acids RNA PMID: 33194561 Poly (I:C) PMID: 31415868 Plasmid PMID: 7779915 DNA PMID: 8717385 Ribosomal preparation PMID: 7325439	Recombinant proteins Heat shock proteins PMID: 12516564 Erythropoietin PMID: 444662 Plasminogen kringle 5 PMID: 14579737 SARS-CoV-2 Spike PMID: 35015170 Human growth hormone PMID: 6386853 Adiponectin PMID: 22374965 C-reactive protein (CRP) PMID: 16254214 Cytokines PMID: 827473 Osteopontin PMID: 15871665
Natural products Natural plant products PMID: 33132322 Glutathione PMID: 29673413 Alginate PMID: 16265647 Chitosan PMID: 37050208 Cotton PMID: 16722191 Pollen PMID: 23238878	Enzymes CRISPR-Cas9 PMID: 34744169 Pancreatic elastase PMID: 15879145 Collagenase PMID: 9930943 Enzyme conjugates PMID: 6348074 Trypsin PMID: 17910052 Lysozyme PMID: 10656326 Superoxide dismutase and catalase PMID: 15251190	
Sugars 3'-Sialyllactose PMID: 29281012 Hyaluronan PMID: 27061945 Cane sugar PMID: 526016 Heparin PMID: 23586950		

Figure 3. Endotoxin contaminations are diverse and ubiquitous. List of publications with pubmed identifiers dealing with recognized endotoxin contamination of various products that can be encountered in daily life (A, *in vivo*) or used during biological experiments (B, *in vitro*).

risk of endotoxin contamination and artefactual results in innate immunity studies, since the reused tips will not be washed with endotoxin-free water.

Immune cells are extremely sensitive to endotoxins

Endotoxins are potent activators of the innate immune system through activation of TLR4 (11, 72), even at very low doses. Interestingly, upon LPS activation, TLR4 is the only TLR that can signal through two different pathways: MyD88 at the plasma membrane and TRIF upon endocytosis (Fig. 4) (73, 74). This results in nuclear factor (NF)- κ B and interferon regulatory factor (IRF)3 translocations, triggering gene

transcription leading to strong inflammatory and antimicrobial responses (75). LPS recognition is further enhanced by several coreceptors: the LBP that transfers monomeric LPS to the CD14 (48, 76, 77) that then shuttles the endotoxin to the MD2 coiled within the TLR4 molecule (78, 79). In the case of *E. coli* LPS, it was reported that the threshold to induce cytokine production in macrophages and dendritic cells is <100 pg/ml, and the threshold to upregulate costimulatory molecules <500 pg/ml (80–82).

In addition to induce cytokine production, LPS are also involved in numerous other innate immune pathways and have been associated with cellular stresses (oxidative stress, autophagy and so on) and even cell death (83–86). Importantly, it is

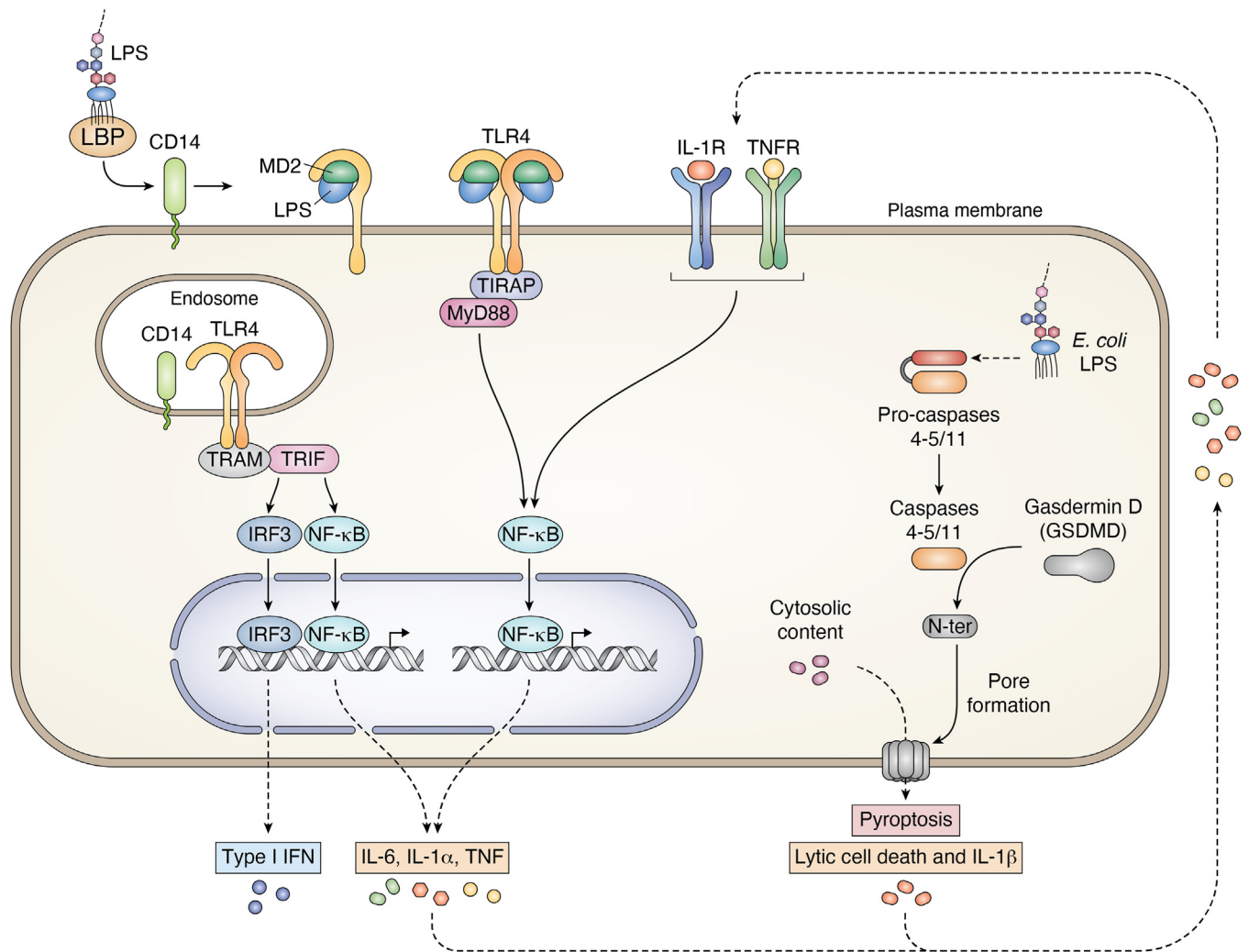


Figure 4. LPS signaling through TLR4 and inflammatory caspases. Diagram of lipopolysaccharide (LPS) signaling in macrophages. LPS first binds to the serum LPS-binding protein (LBP), is delivered to CD14 then transferred to membrane or endosomal TLR4/MD2 complex, which initiates a signaling cascade through the MyD88 and TRIF adapters with the translocation in the nucleus of NF-κB and IRF3 transcription factors, leading to expression of cytokines (type I interferons, or inflammatory cytokines (IL-1β, TNF, and IL-6)). In addition, the LPS is recognized within the cytosol by the caspase 4 or 5 (human), or 11 (mouse) that triggers the caspase dimerization and cleavage of gasdermin D (GSDM), with N terminal parts assembling in large pores, leading to lytic cell death called “pyroptosis”, since it allows a massive release of cytosolic IL-1β, a central cytokine in inflammation provoking fever. Both IL-1β and TNF can amplify the inflammation through retroactivation of their receptors IL-1R and TNFR, also leading to NF-κB activation. IL, interleukin; IRF3, interferon regulatory factor 3; MD2, myeloid differentiation factor 2; TLR, toll-like receptor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

now widely accepted that the LPS can be recognized in host cell cytosol through inflammatory human caspases 4/5 or murine caspase 11 (87–90). This recognition triggers the so-called “noncanonical” inflammasome that results in a lytic cell death called pyroptosis (Fig. 4) (91). Pyroptosis is associated with membrane damage and permeabilization resulting in massive inflammatory burst due to the release of potent cytokines such as IL-1β and IL-18 (92). Overall, endotoxins are not only potent inflammatory mediators, but they also exert strong cytotoxic effects on immune cells and potential contaminations can therefore have a wide range of impacts.

Adjuvant role of endotoxins

Undesired effects may be linked to endotoxin or other *E. coli* contaminations when recombinant proteins are used as drugs, such as recombinant growth hormone or erythropoietin (Fig. 3B). However, the use of *E. coli* recombinant proteins as

subunit vaccines may be of interest, since contaminations may trigger TLR2, TLR4, nucleotide-binding oligomerisation domain receptor (NOD)1, NOD2, and other PRRs. Indeed, TLR and NOD-like receptors ligands are known to be crucial adjuvants to mount an efficient adaptive immune response, and they are already largely used in licensed vaccines (93). However, only synthetic analogs and nontoxic mimetics are used for human vaccines, such as the monophosphoryl Lipid IV_A (94).

Issues about endotoxin contaminations in recombinant proteins

Endotoxins cannot be completely neutralized during purification

In addition to their high ability to activate innate immune cells, endotoxins are almost impossible to remove after protein

expression in *E. coli* (95). Indeed, as described above, endotoxins can bind numerous proteins, in both specific and nonspecific manners (96). These risks are considerably higher when expressing recombinant nonpolar proteins or membrane proteins that are by nature very hydrophobic and therefore most susceptible to bind endotoxins. To address this issue, numerous studies on recombinant proteins have used commercially available solutions to “remove” endotoxins from protein samples, for instance in the *Leptospira* field (see [Examples from the leptospiral field](#) section). Most of them rely on endotoxin removal columns that use PMB coated gels to retain LPS and purify protein samples. Authors treat their samples with PMB, a cationic antimicrobial peptide with LPS-binding affinity, and then state that they have excluded the possibility that contaminating endotoxins may be responsible for the observed phenotypes. We believe that this is not the case, and several studies support this hypothesis. First, one must keep in mind that PMB neutralizes LPS through induction of its aggregation in large micelles, making it less available for receptors and therefore less biologically active. Among other factors, PMB efficiency depends on the intrinsic structural properties of endotoxins, which differ from one bacterium to another, as we previously demonstrated (97, 98) (see [Structure and variability of LPSs](#) section). Interestingly, a study reported that PMB neutralization is only efficient on high concentrations of *E. coli* LPS (3 µg/ml), but not on lower concentrations of LPS (300 ng/ml) (99). Additionally, the coreceptors of TLR4 signaling pathway are known to be able to uptake monomeric LPS moieties from micelles to render them available for TLR4 signaling (48, 76, 77), therefore potentially hindering PMB neutralizing effect. Consistently, another study reported that PMB was not very efficient at preventing the production of proinflammatory cytokines in response to low doses of LPS (80). Also, PMB induces a mild reduction of the LPS negative charges (that are essential for proper signaling) but never completely neutralizes them (99), therefore again only partially reducing endotoxins’ ability to activate host receptors. Finally, several studies have reported that PMB itself could promote the expression and production of proinflammatory cytokines and costimulatory molecules (80, 100, 101). Taken together, these results corroborate that endotoxins removing kits can never guarantee less than 5 EU/ml residual endotoxin, which corresponds roughly to 0.5 to 1 ng/ml, a concentration that is sufficient to activate innate immune response.

Assessment of endotoxin contamination by the LAL assay is not sufficient

To quantify contaminating endotoxins in recombinant proteins, the commercially available *Limulus* amoebocyte lysate (LAL) assay is often used. The LAL is extracted from horseshoe crab’s hemolymph that contains serine proteases which coagulate in cascade in presence of endotoxins, hence allowing their detection. Although this assay is very useful, it presents few drawbacks that impede its efficiency at detecting LPS contaminations. First, the LAL assay is not as sensitive as some immune

cells in sensing LPS. Indeed, a study reported that LAL assay is two orders of magnitude less sensitive than human macrophages stimulated with LPS (81), suggesting that this assay is not sufficient by itself. Second, another drawback of the LAL assay is that the coagulation is not always correlated with the LPS concentrations (102). For instance, several studies reported that some residues in the recombinant proteins, namely hydrophobic residues, may induce LPS binding and consequently prevent its measurement in the assay (95, 103). We have also observed that the fusion of expressed proteins with poly-histidine tag (HIS-tag) prevents the proper quantification of endotoxins by the LAL assay, with almost no contamination detected at 10 µg/ml, whereas serial dilution of the recombinant protein led to increasing endotoxin detection (*personal communication, C. Werts*). Accordingly, it has been demonstrated that cleavage of the HIS-tag allowed a subsequent removal of endotoxins, preferentially binding to cationic residues (104). Another issue with the amoebocyte lysate assays is the origin of the lysate (American horseshoe crabs *versus* Japanese horseshoe crabs) and the consequent variability. Indeed, differences have been observed between the traditional LAL and the *Tachypleus* amoebocyte lysate, the latter also responding to fungal products from *Candida* spp (105). Because of their extensive use for those assays, horseshoe crabs are now endangered species. Animal friendly LPS quantifying methods have been developed, using recombinant factor C, the first endotoxin sensor from the coagulation cascade of limules, but they all present some technical limitations, like the classical LAL assay (20). Advantages and drawback of these different bioassays have been recently reviewed and authors highlight bacterial LPS heterogeneity as a cause of variability in quantification (20). Overall, we therefore think that the LAL and other assays are very useful but must be interpreted with caution when working with recombinant proteins, especially with HIS-tagged proteins, and cannot alone guarantee complete lack of endotoxin contamination.

Commercial PRR/NF-κB HEK reporter cell lines are also convenient to study PRR/MAMPs interactions, including TLR2, TLR3, TLR4, TLR5, TLR8, TLR9, and other PRRs, usually overexpressed. These cells can be useful to test the lack of various bacterial components (such as lipoproteins, LPS, flagellins, or nucleic acids) which may contaminate the recombinant proteins. MD2-TLR4 expressing cells to test the lipid A activity are less sensitive than LAL. However, whole LPS are better recognized (27). Of note, those cells often also express endogenous low levels of other PRRs such as TLR5, TLR3, and NOD1 that may synergize with TLR4. Therefore, careful use of these cells should be done since poor controls or endotoxin contamination of reagents may also easily lead to artefactual results. Indeed, the control NF-κB cells also express the endogenous levels of those PRRs, but the lack of TLR4 overexpression may abolish the synergy, potentially leading to the wrong conclusions about the TLR4 use of the recombinant protein tested.

Endotoxins are not always heat-resistant

It is commonly believed that LPS from *E. coli* is a heat-resistant molecule that is not affected by boiling. Several

authors have heated their recombinant protein preparations and have directly excluded that LPS could be responsible when the phenotype was lost upon heating. However, although heat-resistance may be true for aggregated LPS in concentrated preparations, several studies have reported that lower concentrations of *E. coli* LPS (<100 ng/ml) are more sensitive to heat (personal communication, D. Bonhomme and C. Werts), and that boiling did reduce LPS ability to activate production of inflammatory cytokines (80, 106). Also, the heat-sensitivity of the LPS depends on the bacterial origin. Based on this, we believe that concluding on the nature of the signaling molecule (proteic *versus* endotoxic) after heat treatment is not accurate.

Endotoxins may synergize with other agonists

Beside LPS intrinsic ability to stimulate the immune system at detectable concentration, traces amount of LPS can also lead to cellular activation. Indeed, synergy between different PRR agonists has been evidenced using sub activating doses of each agonist that alone were not able to activate cells but when combined triggered cytokine activation. Hence, sub active doses of LPS synergize with lipoproteins (TLR2 agonists) (107, 108) and with muropeptides (nucleotide oligomerisation domain receptor (NOD) 1 and NOD2 agonists) to activate dendritic cells and macrophages (109). These components are present in *E. coli* and may contaminate the recombinant proteins upon purification. In addition, subactive doses of LPS contaminating plasmids used to produce virus stocks have been shown to synergize with cytosolic DNA sensed by cyclic GMP-AMP synthase (cGAS) and enhance type I interferon (110). Incidentally, these synergistic properties explain the highest potency of commercial crude LPS preparations, compared to repurified LPS, devoid of TLR2, NOD2 and NOD1 activities (111).

Relevant examples from the literature

Since the discovery of TLRs in 1996, numerous studies using recombinant proteins have described host-derived molecules to be TLR2 or TLR4 agonists (Fig. 5) (112). Most of these compounds are secreted upon danger (heat shock proteins (HSPs), high mobility group box1, surfactant), or reside in the extracellular matrix (fibronectin and surfactant protein A). In the same intent to uncover their observed inflammatory effects, the TLR specificity of recombinant proteins from viruses and other microbes were also described. The confusing early years (1996–2010) of deciphering PRR agonist specificities is now over, and the specific recognition of lipid A by MD2-TLR4 and lipoproteins by TLR2 is now well established with crystal structures (113, 114). Other agonists of TLR4/MD2 have been discovered; however, they are either synthetic (115, 116) or endogenous ligands with short acid chains (117). The lack of common structures between the genuine ligands and putative recombinant proteins has called into question the mechanism of their TLR4 activation, which in some cases has been dismissed after appropriate controls or repurification to remove endotoxin. We will present a few examples below.

Endotoxin contamination of HSPs

Both bacterial and mammalian recombinant heat shock proteins (rHSPs) have been shown to trigger inflammation in endothelial and innate immune cells (118) (Fig. 5). Mammalian HSPs of different molecular weights are considered as endogenous danger signals, released upon infection or stress. rHSPs are immunogenic, activate dendritic cell maturation, and elicit T-cell mediated responses. rHSPs, acting as self-adjuvant, have successfully been used as carriers or in fusion with antigen peptides to deliver them to the major histocompatibility complex class I pathway of antigen presenting cells.

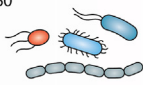
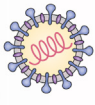

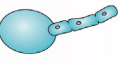

However, the striking parallel between their inflammatory responses *via* TLR2 and TLR4, closely mimicking responses to crude LPS, put into question the mechanism of stimulation. It was found that HSPs can bind LPS with high affinity (118). It has also been shown that removal of endotoxin, testing in TLR4 deficient mice, or direct HSP purification from the mouse liver, resulted in loss of adjuvant effect, suggesting that the high immunogenicity of rHSPs was mainly due to endotoxin contamination (119). This was confirmed in a study correlating endotoxin contamination levels of rHSP70 with their biological effects (120). However, rHSP72, the inducible form of human HSP70, produced in insect cells using a baculovirus system, and therefore endotoxin-free, retained its chaperon effect and activated cytokine production in mouse splenocytes. It may be hypothesized that the very high dose (100 µg) of rHSP70 used to stimulate the cells could give way to baculovirus contaminants, activating the innate immune system (121). Nevertheless, HSPs have many functions and bind to several receptors, such as scavenger receptors to favor endocytosis (122), and the triggering receptor expressed in myeloid cells 2 to enhance phagocytosis (123), both participating in innate immunity.

Driven by studies describing inflammatory properties of eukaryotic HSP, many bacterial HSPs (HSP60 also known as GroEL, HSP65 and HSP70/DNAK) have been described in the early 2000s, as inflammatory molecules (118). For proteins directly purified from Gram-negative bacteria (*e.g.*, *Legionella* and *E. coli*), the LPS present in their membrane, or other MAMPs are likely to have contaminated the proteins. Recombinant GroEL of *Leptospira* has recently been shown to activate mouse macrophages (124) (see [Examples from the leptospiral field](#) section) and recombinant HSP60 from *Chlamydiae* has been described as a TLR4 agonist. In the latter study, the authors excluded the endotoxin contamination, because the endotoxin levels measured with the LAL were very low, and the activation was heat sensitive. However, in both studies, the proteins were expressed as HIS-tagged recombinant proteins in *E. coli*. These confounding factors have been discussed in [Assessment of endotoxin contamination by the LAL assay is not sufficient and Endotoxins are not always heat-resistant](#) sections.

Viral proteins from SARS-COV-2, DENV, and HIV1

The severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) responsible for the COVID-19 pandemics has

Microbial associated molecular patterns

Bacteria	
Endotoxin of Gram-negative bacteria PMID: 9851930	
Fimbriae PMID: 18981086	
Pertussis toxin PMID: 15557205	
HSP60 PMID: 11402040 / PMID: 14573621	
<i>Pseudomonas aeruginosa</i> mannuronic acid polymers PMID: 12089142	
Lumazine synthase PMID: 16455994	
<i>Helicobacter pylori</i> HP0175 PMID: 18806258	
<i>Streptococcus pyogenes</i> OK 432 PMID: 12591988	
<i>Francisella tularensis</i> DnaK PMID: 18708593	
Virus	
Envelope proteins from respiratory syncytial virus (RSV) PMID: 22535679	
Mouse mammary tumor virus (MMTV) PMID: 11854525	
SARS-CoV-2 Spike protein PMID: 33742149	
Dengue virus and Ebola virus glycoprotein PMID: 36246240	
Parasites	
<i>Trypanosoma cruzi</i> glycoinositolphospholipids PMID: 17617606	
Malarial peroxiredoxin PMID: 18398934	
Glycoinositolphospholipids from <i>Leishmania braziliensis</i> and <i>L. infantum</i> PMID: 22389743	
<i>L. mexicana</i> lipophosphoglycan PMID: 24512642	
<i>L. amazonensis</i> lipophosphoglycan PMID: 27508930	
Schistosoma larvae PMID: 16186163	
Fungi	
<i>Cryptococcus neoformans</i> glucuronoxylomanann PMID: 15322035	
Malarial peroxiredoxin PMID: 18398934	
<i>Candida albicans</i> / <i>Saccharomyces cerevisiae</i> mannan PMID: 12222939	
<i>L. mexicana</i> lipophosphoglycan PMID: 24512642	
<i>Taxomyces andreae</i> / <i>Nodulisporium sylviforme</i> paclitaxel (Taxol) PMID: 10644670	
Plants	
Rutin PMID: 35807562	
<i>Angelica gigas</i> Nakai angelan PMID: 17161820	
Lignin-rich enzyme PMID: 25548274	
<i>Arabidopsis thaliana</i> / <i>Nicotiana benthamiana</i> HSP90 PMID: 21701588	
<i>Securidaca inappendiculata</i> Hassk xanthone-rich fraction PMID: 32468498	
<i>Oenanthe javanica</i> pathogenesis-related proteins PMID: 31902921	

Host derived ligands

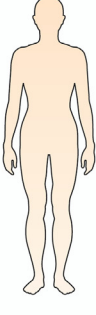
Proteins and peptides		
Alpha-crystallin PMID: 16709864		
Beta-defensin 2 PMID: 12411706		
Heat shock protein GP96 PMID: 16184767		
HSP72 PMID: 17947709		
HSP70 PMID: 11836257		
HSP60 PMID: 10623794		
Resistin PMID: 19754671		
S100A8 and A9 proteins PMID: 17767165		
Surfactant protein A PMID: 12055204		
Fibrinogen PMID: 11509636		
Amyloid beta-peptide PMID: 17986235		
Serum amyloid A PMID: 2385803		
Heat shock protein B8 (HSP22) PMID: 16709864		
Tryptophanyl-tRNA synthetase PMID: 27748732		
High mobility group box 1 protein PMID: 16267105		
Cold-inducible RNA-binding protein PMID: 24097189		
The extra domain A of fibronectin PMID: 11150311		
Granulysin PMID: 20660289		
α 1-microglobulin and α 2-macroglobulin PMID: 22225630		
Xanthine oxidase PMID: 18287332		
Sugars / proteoglycans		
Biglycan PMID: 16025156		
Fibrinogen PMID: 11509636		
Heparan sulphate PMID: 22188870		
Hyaluronan PMID: 11781369		
Tenascin-C PMID: 19561617		
Lipids and associated molecules		
Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine PMID: 12777373		
Gangliosides PMID: 16651628		
Lauric acid PMID: 12865424		
Minimally modified low density lipoprotein PMID: 12424240		
Oxidized low density lipoprotein PMID: 20467793		
Others		
Monosodium urate crystals PMID: 16142712		
Heme PMID: 17502383		
Nickel, cobalt, palladium PMID: 23692033		

Figure 5. TLR4/MD2 real & putative ligands. List of studies and corresponding PMIDs describing microbial associated molecular patterns (MAMPs) from bacteria, parasites, virus, and plants (*left box*) and host derived proteins, sugars, and lipids (*right box*) as putative TLR4 agonists. In **bold**, endotoxin from Gram-negative bacteria is the real TLR4 agonist. MD2, myeloid differentiation; TLR, toll-like receptor.

been the subject of intense research, particularly in the field of innate immunity. The fact that severe symptoms observed in patients mimic certain features of bacterial sepsis prompted researchers to investigate the causes of hyperinflammation, sometimes referred to as “cytokine storm”, although unlike sepsis, it is not systemic but localized in the lungs (125). Using recombinant protein produced in *E. coli* or in human HEK293 cells, the SARS-COV-2 spike S1 subunit, which binds the angiotensin-converting enzyme 2 receptor on mammalian cells, has been shown to be a key inflammatory mediator and has been considered a TLR4 agonist in macrophages (126, 127). Interestingly, another study using recombinant spike protein produced in human epithelial HEK293 cells did not show activation by TLR4, but by the TLR2/TLR6 receptor of diacylated lipoproteins (128). In the latter, it is plausible that

the effect attributed to the recombinant spike protein could be related to mycoplasmas, since they are TLR2/6 agonists and are a frequent cause of contamination of cell lines (129). Of note, in a more recent article entitled “TLR2 senses the SARS-CoV-2 envelop protein to produce inflammatory cytokines”, the authors used recombinant proteins to show that the envelop but not the spike protein, stimulates TLR2 (130). However, they do not discuss the fact that the Env is a 6xHis-tagged protein expressed in *E. coli*, whereas the spike is produced in HEK293 cells, and therefore is devoid of MAMPs.

Finally, a study reexamined in primary human macrophages the responses to five different recombinant spike S1 proteins produced in *E. coli* or human cells, and showed that endotoxin contamination, and not the glycosylation state, was responsible for the artifactual inflammation observed (131). Interestingly, a

study demonstrated that a recombinant SARS-COV-2 spike protein binds LPS with high affinity, leading to enhanced LPS inflammatory activity (132). Indeed, the authors showed that a HIS-tagged version of the spike protein produced in HEK293 cells, that by itself was not inflammatory, induced a measurable inflammation in THP-1-CD14 reporter cells when mixed with a minimal amount of 2.5 ng/ml of LPS, although the same amount of LPS alone did not induce inflammation (132). The authors subsequently showed by molecular docking that the LPS would bind the S1 subunit part of the protein. However, it would have been very interesting to check whether the spike protein devoid of the HIS-tag could also bind the LPS with the same affinity, since LPS exhibits high affinity to stretches of cationic histidine residues (104) (see [Assessment of endotoxin contamination by the LAL assay is not sufficient section](#)).

The high number of publications showing that the SARS-COV-2 protein stimulate TLRs pushed forward *in silico* approaches to better understanding the interaction between the spike protein and TLRs. The SARS-COV-2 spike protein has been shown by molecular docking to bind to the external part of leucine rich repeats domains of human TLR4, TLR2, and TLR1 (133), whereas another study found potent interactions of several spike S1 epitopes with the inner part of TLR4 leucine rich repeat and MD2 (134). These studies also aimed to propose new therapeutic strategies to decrease the inflammation upon SARS-COV-2 infection, with peptides targeting the regions of interaction of spike and TLR4. Most probably these projects based on weak assumptions may be not successful and may even be problematic considering the cornerstone role of TLR4 in host defense.

Dengue is a viral disease transmitted by female *Aedes* sp. mosquito. Dengue viruses (DENV) are flaviviruses that replicate in dendritic cells, monocytes, and macrophages. The hallmark of severe dengue is vascular permeability and consequent plasma leakage, potentially leading to circulatory collapse, shock, and death. High levels of circulating inflammatory cytokines and chemokines correlate with severe outcomes observed in dengue patients with hemorrhagic fever (135). Nonstructural (NS)1 protein is one of the 7 NS proteins involved in intracellular replication and genome packaging but is also secreted by infected cells as a hexamer found in blood circulation. NS1 plays a pivotal role by subverting the complement system (136). Recombinant NS1 has been incriminated in the inflammatory effects, endothelia permeability (137), and platelet activation (138), and was further shown to be either a TLR4 (138, 139) or TLR2/6 agonist (140). Using macrophages from TLR-deficient mice and TLR-blocking antibodies, the authors of the first study reexamined the inflammatory properties and TLR utilization of recombinant NS1 proteins from different sources: *E. coli* with HIS-TAG (140), stably transfected *Drosophila* S2 cells, or baculovirus insect system (138, 139), and compared these recombinant proteins with native NS1 purified from DENV-infected Vero cell supernatants (141). Only the latter formed a hexameric structure, without apparent endotoxin contamination, and activated TLR4, suggesting that TLR4 may indeed be an NS1 receptor. Consistently, platelet activation was shown *ex vivo*

with supernatants from cells infected with DENV but not Zika virus (138), and reduced thrombocytopenia has been evidenced in a TLR4-deficient DENV mouse model (138, 139). Dengue fever is a global threat still awaiting specific antiviral treatments. Although these studies received much attention in the hope of developing therapeutic TLR4 antibodies to treat DENV infection, the authors honestly state that “It is still conceivable that trace amounts of LPS within tissue culture reagents could be bound and concentrated on NS1 in a manner inaccessible to polymyxin B and the Limulus amoebocyte lysate assay, with NS1 becoming an efficient delivery agent for LPS to the TLR4 receptor complex” (141). Indeed, the fact that DENV is a stealth pathogen that has evolved numerous mechanisms to evade innate and adaptive immune responses (136) argues against an inflammatory role for NS1. In particular, DENV NS proteins are involved in modulating and escaping the type I interferon response, by blocking at different levels the TLR3, RIG-I, cGAS, and STING pathways (136). In fact, it has been shown that leaky gut is associated with severe dengue. Consistent with gut translocation of large molecules, higher serum levels of LPS and β -glucan were measured in severe dengue patients compared with nonsevere dengue patients, which may explain the “cytokine storm” and endotoxemia (64).

The HIV-1 virus field is not exempt of confounding effects of LPS. Indeed, several HIV-1 recombinant proteins have been described as TLR4 agonists. First, the recombinant virion-associated protein of unknown function was reported to activate TLR4/MyD88-mediated IL-6 production and reactivate viral production from latency (142). Then, the envelope glycoprotein gp120 which is important for virus entry by binding to human T-cells CD4 and CCR5 receptors, and that is shed in large quantities in serum, has been shown to activate genital epithelial cells, and further shown to stimulate inflammation through binding to both TLR2 and TLR4 (143). This study was performed with recombinant gp120, expressed in a baculovirus expression system, and commercially sold as endotoxin-free. Of note, the TLR response required the presence of heparan sulfate, a compound that is known to enhance TLR4 responses (144) (Fig. 5). The same group reported the same year, that recombinant transactivator of HIV1 gene expression was also a TLR4-MD2 agonist (145). In that case, recombinant transactivator was a glutathione-S-transferase fusion protein expressed in *E. coli* which was considered endotoxin free despite an endotoxic level measured by LAL of 0.3 EU/ μ g. Those three examples of different HIV proteins activating through TLR4 should be reevaluated. It is established that HIV1 activates cells and induces proinflammatory cytokines and type I interferon through classical viral MAMPs, such as RNAs recognized by endosomal TLR3/7/8 and cytosolic RIG-I, as well as retro-reversed transcribed DNA sensed by the cGAS/STING pathway. In addition, gut damage and microbial translocation occur in HIV1 patients, with measurable levels of serum LPS, CD14 and other microbial inflammatory components such as flagellin or β -glucan (146), which may explain the chronic inflammation observed in people living with HIV1, despite effective antiviral treatments (147).

Examples from the leptospiral field

Leptospire are stealth bacteria that escape many innate immune recognition pathways (148). In particular, because of its peculiar structure (26), the LPS of leptospire is not a human TLR4 agonist (53). However, it is recognized by mouse TLR4 (27), but only triggers a mild response in mouse macrophages. Indeed, the leptospiral LPS signals through the MyD88 pathway but avoids the TRIF pathway (54). The fact that whole leptospire do not signal *via* human TLR4, even when degraded by heating or lysed, should warn against data showing recombinant leptospire proteins to be human TLR4 agonists. Moreover, even if leptospire can enter and reside in macrophages (149), they do not induce cell death in mouse, human, bovine, and hamster macrophages (150). Indeed, the leptospiral LPS does not trigger the “noncanonical” inflammasome, which limits the release of the potent IL-1 β cytokine (150) (see [Issues about endotoxin contaminations in recombinant proteins](#) section). Therefore, even minor endotoxin contamination in recombinant proteins expressed in *E. coli* may lead to erroneous conclusions on the putative inflammatory or lytic roles of these leptospiral proteins. Considering the endotoxin contamination issues described above in [Issues about endotoxin contaminations in recombinant proteins](#) and [Relevant examples from the literature](#) sections, we reviewed different studies using recombinant *Leptospira* proteins (124, 151–159). For each article, we present in [Table 1](#), the factsheet of the study and critically assessed the potential endotoxin contamination and conclusions about the immune functions and lytic properties of the studied recombinant proteins.

Other solutions for recombinant proteins expression and purification

All microbial-based expression systems must be used with caution

An option to avert endotoxin contamination is to use the ClearColi *E. coli* expression system, in which bacteria do not possess a classical LPS, but only have a tetra-acylated Lipid_{IV}A (160). The main advantage of this system is that Lipid_{IV}A is not an agonist of human-TLR4/MD2 complex (161), therefore preventing the risk that immune cells may be activated by contaminants. Although this system can be used to study the response of human cells, it is not appropriate for other species because Lipid_{IV}A is still an agonist for less stringent receptors such as mouse-TLR4, hamster-TLR4, and horse-TLR4 (161, 162). To overcome the endotoxin issue, LPS-free bacteria may also be used as protein expression platforms, such as Gram-positive *Bacillus subtilis* (163), or *Sphingomonas*, in which LPS are replaced with nontoxic glycosphingolipids (164). However, in these systems, other bacterial contaminants and PRR agonists, such as lipoproteins, lipoteichoic acids, muropeptides, or DNA can contaminate the recombinant proteins, which should also limit their use as therapeutics or in innate immunity.

Another possibility is to use plant expression systems that present the advantage to allow the production of recombinant glycoproteins (165). Active recombinant human and mouse

cytokines have been successfully produced in rice seeds, with low endotoxin contamination (166). However, because of the symbiotic association of the plants with microbes, we may expect the same issues as described above and should be very cautious regarding bacterial contamination of recombinant proteins.

Yeasts may also be used as protein expression platforms since they allow posttranslational glycosylation (167). Here, the issue may be contamination with β -glucans, agonists of dectins (168), that potently synergize with TLRs and modulate immune responses (169).

Proteins may be expressed in other systems

The safest choice to express recombinant proteins intended for innate immunity studies is to either use insect cells or mammalian expression systems, both devoid of any MAMPs. However, in the case of insect cells, the use of replicative plasmids (prepared with kits allowing low endotoxin content) should be preferred over baculovirus expression systems. Although they are powerful and versatile systems for producing high levels of recombinant protein expressions, baculovirus virus DNA and particles that also trigger innate immunity in mammalian cells, may contaminate the recombinant proteins.

Finally, cell-free expression mammalian-based systems using human lysates may allow rapid synthesis of recombinant proteins with native posttranslational modifications without microbial contamination. For small proteins, synthetic proteins could also be a good alternative. One should also recall that putative LPS contaminations can occur during the purification process.

Conclusion

Endotoxins are a major issue when studying innate immunity. They can contaminate culture reagents and recombinant proteins. The risk associated with the use of recombinant proteins in innate immune studies is the erroneous conclusion that many proteins, sugars, and lipids could activate the innate immune cells *via* the well-characterized TLR4 receptor of LPS. This also applies to the innate study of bacteria, parasites, and fungi derived products. In the *Leptospira* field, close examination of whether the conclusions of the studies seemed appropriate depends on the expression systems used to generate the recombinant protein. Whether the observed cellular phenotype could be attributed to LPS has to be very carefully examined. It is important that researchers are aware of the confusing effects of endotoxin bound to their protein. To avoid such problems, we have presented several expression and purification methods that may help readers to plan their future experimental setup. Not all recombinant proteins can be TLR4/MD2 agonists. Indeed, a red flag should be put on recombinant proteins described as TLR4 agonists, especially those constructed with a HIS-tag.

Even if these studies using recombinant proteins were carried out on good faith, they pollute the field of innate immunity. Modeling studies using sophisticated softwares and

Table 1
Studies on leptospiral recombinant protein with potential endotoxin contaminations

Reference	Protein	Expression system	Quality control	[X] max	Immune effects observed with REC-proteins	Compatible with contaminations
Che <i>et al.</i> , 2019 PMID: 30278102	Sph2 Hemolysin from <i>L. interrogans</i> serovar Lai	Bacteria <i>E. coli</i>	None provided presence of HIS-Tag	10 µg/ml ~70 kDa ~150 nM	1. rec-Sph2 induces cell death With LDH release With annexinV ⁺ /PI ⁺ staining 2. rec-Sph2 induces production of ROS With alteration of mitochondrial potential	1. Yes Lytic cell death (pyroptosis) is classically triggered by internalized LPS. 2. Yes LPS-TLR4 induces ROS production.
Faisal <i>et al.</i> , 2016 PMID: 27996041	Lsa21 Adhesin from <i>L. interrogans</i> serovar Pomona	Bacteria <i>E. coli</i>	1. DetoxiGel (Pierce) endotoxin removal <i>NB: supplier cannot guarantee less than 5EU/ml (~1 ng/ml) residual LPS</i> 2. LAL test (Biomedicals) Results not provided	2 µg/ml ~21 kDa ~100 nM	1. rec-Lsa21 activates HEK-TLR2 cells 2. rec-Lsa21 triggers TNF & IL6 production TLR2/TLR4-dependent 3. rec-Lsa21 induces costimulatory & immune genes TLR2/TLR4-dependent	1. Maybe HEK do not express TLR4 but express endogenous TLR5 and NOD1 that might sense other contaminants (flagellin/PG) from <i>E. coli</i> . 2/3. Yes LPS-TLR4 induce inflammatory genes upregulation.
Fang <i>et al.</i> , 2018 PMID: 30337247	vWAI vWAI Proteins with vWF domains from <i>L. interrogans</i> serovar Lai	Bacteria <i>E. coli</i>	1. DetoxiGel (Pierce) endotoxin removal <i>NB: supplier cannot guarantee less than 5EU/ml (~1 ng/ml) residual LPS</i> 2. LAL test (Lonza) Results suggesting <0.1 ng/ml but presence of HIS-Tag	5 µg/ml ~36 kDa ~140 nM	1. rec-vWAI/II do not trigger platelets aggregation 2. No other form of platelets activation described Including no NO production 3. rec-vWAI/II inhibit vWF induced platelets aggregation	1/2. No Negative phenotypes (especially NO production) do not suggest contaminations with LPS. 3. No LPS role is controversial but there is no strong evidence suggesting it could prevent platelet aggregation.
Hsu <i>et al.</i> , 2020 PMID: 33094809	LRR20 Leucine reach repeat 20 protein from <i>Leptospira santarosai</i> serovar <i>Shermani</i>	Bacteria <i>Escherichia coli</i> "ClearColi" (lipid _{IV} A)	None provided presence of HIS-Tag	200 µg/ml ~20 kDa ~10 µM	1. rec-LRR20 presents specific affinity for E-cadherin No affinity for TLR2 presented as negative control 2. rec-LRR20 induces NGAL in HK2 (kidney cells)	1. No LPS is not accountable for protein/protein interactions. 2. No HK2 proximal tubular cells do not express TLR4 at basal levels and use of ClearColi dismiss TLR4 activation.
Hsu <i>et al.</i> , 2021 PMID: 33441663	Loa22 Lipoprotein from <i>L. santarosai</i> serovar <i>Shermani</i>	Bacteria <i>E. coli</i> "ClearColi" (lipid _{IV} A)	1. MonoQ column with polymyxin B 2. LAL test Results suggesting <<0.05 ng/ml but presence of HIS-Tag	100 ng/ml ~22 kDa ~5 nM	1. rec-Loa22 with PG activates HEK-TLR2 cells 2. rec-Loa22 with PG activates PMA-induced THP1 cells	1. No HEK do not express basal TLR4 and it is expected that lipoproteins activate TLR2. 2. No THP1 express huTLR4 which is not sensitive to lipid _{IV} A from ClearColi <i>E. coli</i> .
Kumar <i>et al.</i> , 2021 PMID: 34975922	LigA Surface protein from <i>L. interrogans</i> serovar Pomona	Bacteria <i>E. coli</i>	1. DetoxiGel (Pierce) endotoxin removal <i>NB: supplier cannot guarantee less than 5EU/ml (~1 ng/ml) residual LPS</i> 2. LAL test (Pierce) Results not provided presence of HIS-Tag	2 µg/ml ~73 kDa ~30 nM	1. rec-HIS-LigA variable region (LAV) activates macrophages Proteinase K sensitive & polymyxin B resistant 2. rec-HIS-LAV activates HEK-TLR4 cells 3. rec-HIS-domain11 is involved in the signaling 4. rec-HIS-LAV triggers protective IgG1 & IgG2c response 5. rec-HIS-LAV prevents complement mediate killing Binding to factor H hence triggering C3b cleavage	1/2/3. Yes LPS-TLR4 might potentiate the response to rec-LAV. 4. Yes IgG2c antibodies suggest that at least part of the response is LPS-mediated. 5/6. No LPS is not accountable for the enzymatic activities of rec-LAV (although it could potentiate its effects).

Table 1—Continued

Reference	Protein	Expression system	Quality control	[X] max	Immune effects observed with REC-proteins	Compatible with contaminations	
Kumar <i>et al.</i> , 2022 PMID: 35401498	LenB LenD LenE Lsa30 Loa22 LipL21	Lipoproteins from <i>Leptospira interrogans</i> serovar Pomona	Bacteria <i>E. coli</i>	Estimated endotoxin (no method indicated) 0.10–0.15 ng/ml but presence of HIS-Tag	1 µg/ml ~21–65 kDa ~15–50 nM	6. rec-HIS-LAV prevents NET formation upon infection With efficient DNase activity 1. rec-HIS-lipoproteins induces inflammatory responses Production of TNF and IL6 Upregulation of costimulatory molecules Activation is polymyxin B resistant 2. cow and human macrophages respond less than mouse 3. rec-HIS-lipoproteins bind complement FH and C4BP 4. rec-HIS-lipoproteins act as nucleases and degrade NETs	1. Yes Observed effects cannot be mediated by TLR2 since only the protein part of lipoproteins are expressed and lipoproteins needs the acyl chains to signal via TLR2. The stimulation could be induced by TLR4/LPS. 2. Yes Species-specificity for TLR4 are described. 3/4. No LPS is not accountable for protein/protein interactions nor for enzymatic activities (although it could potentiate their effects).
Passalia <i>et al.</i> , 2020 PMID: 32078713	BatA (vWAI) BatB (vWAI1)	Proteins with vWF domains from <i>L. interrogans</i> serovar Copenhageni	Bacteria <i>E. coli</i>	None provided	5 µg/ml ~36 kDa ~140 nM	1. rec-BatA is a serine protease 2. rec-BatA/B cleave human fibrinogen 3. rec-BatA/B inhibit platelet aggregation	1/2. No LPS is not accountable for the enzymatic activities of rec-Bats. 3. No LPS role is controversial but there is no strong evidence suggesting it could prevent platelet aggregation.
Wang <i>et al.</i> , 2012 PMID: 22870312	Sph1 Sph2 Sph3 HlpA TlyA	Haemolysin from <i>L. interrogans</i> serovar Lai	Bacteria <i>E. coli</i>	1. DetoxiGel (Pierce) endotoxin removal <i>NB: supplier cannot guarantee less than 5EU/ml (~1 ng/ml) residual LPS</i> 2. LAL test (Pierce) Results not provided but HIS-tag 2. ELISA with anti- <i>E. c</i> LPS IgG (Abcam) Results not provided	10 µg/ml ~30–65 kDa ~150–330 nM	1. rec-HIS-haemolysins do not induce cell death 2. rec-HIS-haemolysins induce IL1β/TNF/IL6 Proteinase K sensitive and polymyxin B resistant Through TLR2 and TLR4 Through JNK and NF-κB	1. No 2. Yes Observed effects could be induced by TLR4/LPS and lipoproteins/TLR2.
Ho <i>et al.</i> , 2021 PMID: 33789603	GroEL	Heat shock protein 60 from <i>L. interrogans</i> Serovar Copenhageni	Bacteria <i>E. coli</i>	1. DetoxiGel (Pierce) endotoxin removal 2. LAL test (Lonza) 2EU/µg (but presence of HIS TAG impairs the LAL test)	10 µg/ml 60 kDa 160 nM	1. rec-HIS-GroEL binds extracellular matrix and proteins 2. rec-HIS-GroEL binds Vero cells 3. rec-HIS-GroEL induces TNF/IL6 in murine macrophages Proteinase K sensitive and polymyxin B resistant	1/2. No LPS is not accountable for the binding activities of rec-GroEL. 2. Yes LPS-TLR4 is responsible for the rec-HIS-GroEL macrophage activation.

For each publication suggesting that a leptospiral protein could be inflammatory or a TLR agonist, are indicated the name of the protein, the expression system, the results of endotoxin detection tests, if the protein has been HIS-tagged (NB the presence of HIS-Tag impairs the detection with the Limulus assay), the concentration used to stimulate cells, and the principal conclusions of the authors. In the last column, we critically assess the authors conclusions and explain why there is no problem since the endotoxin contamination should not interfere with their observations (NOT) or that the conclusions are probably erroneous since the results are compatible with endotoxin contamination (YES), or potentially wrong because of other bacterial contaminations (MAYBE).

artificial intelligence are now being used intensively to predict new TLR4 agonists and to decipher safe vaccines adjuvants. The fact that these *in silico* studies may rely on hundreds of artefactual data based on recombinant protein studies should raise alarm bells.

Author contributions—D. B., J.-M. C., and C. W. writing—original draft; D. B., J.-M. C., and C. W. writing—review and editing; D. B. visualization; C. W. conceptualization; C. W. supervision; C. W. project administration.

Funding and additional information—This work has been funded by Institut Pasteur.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: BPI, bactericidal/permeability increasing protein; CD, cluster of differentiation; cGAS, cyclic GMP-AMP synthase; DENV, dengue viruses; HSP, heat shock protein; IL, interleukin; LAL, Limulus amoebocyte lysate; LBP, LPS-binding protein; LPS, lipopolysaccharides; MAMPs, microbe-associated molecular patterns; MD2, myeloid differentiation factor; NS, nonstructural; PMB, polymyxin B; PRR, pattern recognition receptor; rHSP, recombinant heat shock protein; SARS-COV-2, severe acute respiratory syndrome coronavirus 2; sCD14, soluble cluster of differentiation; TLR, toll-like receptor.

References

1. Janeway, C. A., Jr. (1989) Approaching the asymptote? evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54**, 1–13
2. Matzinger, P. (1994) Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* **12**, 991–1045
3. Cavaillon, J. M., and Legout, S. (2016) Centenary of the death of Elie Metchnikoff: a visionary and an outstanding team leader. *Microbes Infect.* **18**, 577–594
4. Steinman, R. M., and Cohn, Z. A. (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* **137**, 1142–1162
5. Brieger, L. (1888) Zur kenntnis des tetanin und des mytilotoxin. *Arch. Pathol. Anat. Physiol. Klin. Med.* **112**, 549e551
6. Menkin, V. (1944) Chemical basis of fever. *Science* **100**, 337–338
7. Wood, W. B., Jr. (1958) The role of endogenous pyrogen in the genesis of fever. *Lancet* **2**, 53–57
8. Bennett, I. L., Jr., and Beeson, P. B. (1953) Studies on the pathogenesis of fever. II. Characterization of fever-producing substances from polymorphonuclear leukocytes and from the fluid of sterile exudates. *J. Exp. Med.* **98**, 493–508
9. Cavaillon, J. M. (2018) Exotoxins and endotoxins: inducers of inflammatory cytokines. *Toxicon* **149**, 45–53
10. Barratt, G. M., Raddassi, K., Petit, J. F., and Tenu, J. P. (1991) MDP and LPS act synergistically to induce arginine-dependent cytostatic activity in rat alveolar macrophages. *Int. J. Immunopharmacol.* **13**, 159–165
11. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., et al. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085–2088
12. Warren, H. S., Fitting, C., Hoff, E., Adib-Conquy, M., Beasley-Topliffe, L., Tesini, B., et al. (2010) Resilience to bacterial infection: difference between species could be due to proteins in serum. *J. Infect. Dis.* **201**, 223–232
13. Cavaillon, J. M., Singer, M., and Skirecki, T. (2020) Sepsis therapies: learning from 30 years of failure of translational research to propose new leads. *EMBO Mol. Med.* **12**, e10128

14. Libert, C., Ayala, A., Bauer, M., Cavaillon, J. M., Deutschman, C., Frostell, C., et al. (2019) Part II: minimum quality threshold in pre-clinical sepsis studies (MQTiPSS) for types of infections and organ dysfunction endpoints. *Shock* **51**, 23–32
15. Moore, P. K., Anderson, K. C., McManus, S. A., Tu, T. H., King, E. M., Mould, K. J., et al. (2023) Single-cell RNA sequencing reveals unique monocyte-derived interstitial macrophage subsets during lipopolysaccharide-induced acute lung inflammation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **324**, L536–L549
16. Wierenga, K. A., Riemers, F. M., Westendorp, B., Harkema, J. R., and Pestka, J. J. (2022) Single cell analysis of docosahexaenoic acid suppression of sequential LPS-induced proinflammatory and interferon-regulated gene expression in the macrophage. *Front. Immunol.* **13**, 993614
17. Qiu, Y., Yu, P., Lin, R., Fu, X., Hao, B., and Lei, B. (2017) Genome-wide retinal transcriptome analysis of endotoxin-induced uveitis in mice with next-generation sequencing. *Mol. Vis.* **23**, 395–406
18. Cavaillon, J. M. (2020) Andre Boivin: a pioneer in endotoxin research and an amazing visionary during the birth of molecular biology. *Innate Immun.* **26**, 165–171
19. Westphal, O., Luderitz, O., and Bister, F. (1952) Über des extraktion von bacterien mit phenolwasser Z Naturforsch 7b. *Z. Naturforsch. B Chem. Sci.* <https://doi.org/10.1515/znb-1952-0303>
20. Fux, A. C., Casonato Melo, C., Michelini, S., Swartzwelter, B. J., Neusch, A., Italiani, P., et al. (2023) Heterogeneity of lipopolysaccharide as source of variability in bioassays and LPS-binding proteins as remedy. *Int. J. Mol. Sci.* **24**, 8395
21. Cross, A. S. (2023) Hit 'em where it hurts: gram-negative bacterial lipopolysaccharide as a vaccine target. *Microbiol. Mol. Biol. Rev.* **87**, e0004522
22. Caroff, M., and Novikov, A. (2020) Lipopolysaccharides: structure, function and bacterial identification. *OCL* **27**, 10
23. Brandenburg, K., Schromm, A. B., and Gutschmann, T. (2010) Endotoxins: relationship between structure, function, and activity. *Subcell. Biochem.* **53**, 53–67
24. Nghiem, H. O., Staub, A. M., Galanos, C., and Luderitz, O. (1982) Distribution and antigenic properties of the O-determinants of Salmonella zuerich (1, 9, 27 46). *Eur. J. Biochem.* **125**, 431–436
25. Cavaillon, J. M., and Haeflner-Cavaillon, N. (1987) Characterization of the induction of human interleukin-1 by endotoxins lipid mediators in the immunology of shock. *NATO ASI* **139**, 395–407
26. Que-Gewirth, N. L., Ribeiro, A. A., Kalb, S. R., Cotter, R. J., Bulach, D. M., Adler, B., et al. (2004) A methylated phosphate group and four amide-linked acyl chains in *Leptospira interrogans* lipid A. The membrane anchor of an unusual lipopolysaccharide that activates TLR2. *J. Biol. Chem.* **279**, 25420–25429
27. Nahori, M. A., Fournie-Amazouz, E., Que-Gewirth, N. S., Balloy, V., Chignard, M., Raetz, C. R., et al. (2005) Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells. *J. Immunol.* **175**, 6022–6031
28. Kawahara, K., Tsukano, H., Watanabe, H., Lindner, B., and Matsuura, M. (2002) Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. *Infect. Immun.* **70**, 4092–4098
29. Ciornei, C. D., Novikov, A., Beloin, C., Fitting, C., Caroff, M., Ghigo, J. M., et al. (2010) Biofilm-forming *Pseudomonas aeruginosa* bacteria undergo lipopolysaccharide structural modifications and induce enhanced inflammatory cytokine response in human monocytes. *Innate Immun.* **16**, 288–301
30. Hajjar, A. M., Ernst, R. K., Tsai, J. H., Wilson, C. B., and Miller, S. I. (2002) Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat. Immunol.* **3**, 354–359
31. Novikov, A., Breton, A., and Caroff, M. (2017) Micromethods for isolation and structural characterization of lipid A, and polysaccharide regions of bacterial lipopolysaccharides. *Methods Mol. Biol.* **1600**, 167–186
32. Tobias, P. S., Soldau, K., and Ulevitch, R. J. (1986) Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *J. Exp. Med.* **164**, 777–793
33. Frey, E. A., Miller, D. S., Jahr, T. G., Sundan, A., Bazil, V., Espevik, T., et al. (1992) Soluble CD14 participates in the response of cells to lipopolysaccharide. *J. Exp. Med.* **176**, 1665–1671

34. Wurfel, M. M., Kunitake, S. T., Lichenstein, H., Kane, J. P., and Wright, S. D. (1994) Lipopolysaccharide (LPS)-binding protein is carried on lipoproteins and acts as a cofactor in the neutralization of LPS. *J. Exp. Med.* **180**, 1025–1035
35. Heumann, D., and Roger, T. (2002) Initial responses to endotoxins and Gram-negative bacteria. *Clin. Chim. Acta* **323**, 59–72
36. Larrick, J. W., Hirata, M., Zheng, H., Zhong, J., Bolin, D., Cavaillon, J. M., et al. (1994) A novel granulocyte-derived peptide with lipopolysaccharide-neutralizing activity. *J. Immunol.* **152**, 231–240
37. Opal, S. M., Palardy, J. E., Marra, M. N., Fisher, C. J., Jr., McKelligon, B. M., and Scott, R. W. (1994) Relative concentrations of endotoxin-binding proteins in body fluids during infection. *Lancet* **344**, 429–431
38. Evans, T. J., Carpenter, A., Moyes, D., Martin, R., and Cohen, J. (1995) Protective effects of a recombinant amino-terminal fragment of human bactericidal/permeability-increasing protein in an animal model of gram-negative sepsis. *J. Infect. Dis.* **171**, 153–160
39. Levin, M., Quint, P. A., Goldstein, B., Barton, P., Bradley, J. S., Shemie, S. D., et al. (2000) Recombinant bactericidal/permeability-increasing protein (rBPI21) as adjunctive treatment for children with severe meningococcal sepsis: a randomised trial. rBPI21 Meningococcal Sepsis Study Group. *Lancet* **356**, 961–967
40. Cavaillon, J. M., Fitting, C., Haeffner-Cavaillon, N., Kirsch, S. J., and Warren, H. S. (1990) Cytokine response by monocytes and macrophages to free and lipoprotein-bound lipopolysaccharide. *Infect. Immun.* **58**, 2375–2382
41. Munford, R. S., and Hall, C. L. (1986) Detoxification of bacterial lipopolysaccharides (endotoxins) by a human neutrophil enzyme. *Science* **234**, 203–205
42. Cavaillon, J. M., Fitting, C., Hauttecoeur, B., and Haeffner-Cavaillon, N. (1987) Inhibition by gangliosides of the specific binding of lipopolysaccharide (LPS) to human monocytes prevents LPS-induced interleukin-1 production. *Cell. Immunol.* **106**, 293–303
43. David, S. A., Balaram, P., and Mathan, V. I. (1995) Characterization of the interaction of lipid A and lipopolysaccharide with human serum albumin: implications for an endotoxin carrier function for albumin. *J. Endotoxin Res.* **2**, 99–106
44. Gioannini, T. L., Zhang, D., Teghanemt, A., and Weiss, J. P. (2002) An essential role for albumin in the interaction of endotoxin with lipopolysaccharide-binding protein and sCD14 and resultant cell activation. *J. Biol. Chem.* **277**, 47818–47825
45. James, P. E., Jackson, S. K., Rowlands, C. C., and B. M. (1992) Molecular interactions between bacterial endotoxins and mammalian cells. A spin-label study. *J. Chem. Soc. Perkin Trans. 2*, 1503–1508
46. Dziarski, R. (1994) Cell-bound albumin is the 70-kDa peptidoglycan-, lipopolysaccharide-, and lipoteichoic acid-binding protein on lymphocytes and macrophages. *J. Biol. Chem.* **269**, 20431–20436
47. Haeffner-Cavaillon, N., Chaby, R., Cavaillon, J. M., and Szabo, L. (1982) Lipopolysaccharide receptor on rabbit peritoneal macrophages. I. Binding characteristics. *J. Immunol.* **128**, 1950–1954
48. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249**, 1431–1433
49. Cavaillon, J. M., Marie, C., Caroff, M., Ledur, A., Godard, I., Poulain, D., et al. (1996) CD14/LPS receptor exhibits lectin-like properties. *J. Endotoxin Res.* **3**, 471–480
50. Kirschning, C. J., Wesche, H., Merrill Ayres, T., and Rothe, M. (1998) Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med.* **188**, 2091–2097
51. Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., et al. (1998) Toll receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* **395**, 284–288
52. Hirschfeld, M., Ma, Y., Weis, J. H., Vogel, S. N., and Weis, J. J. (2000) Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J. Immunol.* **165**, 618–622
53. Werts, C., Tapping, R. I., Mathison, J. C., Chuang, T. H., Kravchenko, V., Saint Girons, I., et al. (2001) Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat. Immunol.* **2**, 346–352
54. Bonhomme, D., Santicchia, I., Vernel-Pauillac, F., Caroff, M., Germon, P., Murray, G., et al. (2020) Leptospiral LPS escapes mouse TLR4 internalization and TRIF-associated antimicrobial responses through O antigen and associated lipoproteins. *PLoS Pathog.* **16**, e1008639
55. Viriyakosol, S., Tobias, P. S., Kitchens, R. L., and Kirkland, T. N. (2001) MD-2 binds to bacterial lipopolysaccharide. *J. Biol. Chem.* **276**, 38044–38051
56. Douglas, G. W., Beller, F. K., and Debrovner, C. H. (1963) The demonstration of endotoxin in the circulating blood of patients with septic abortion. *Am. J. Obstet. Gynecol.* **87**, 780–788
57. Levin, J., Poore, T. E., Zauber, N. P., and Oser, R. S. (1970) Detection of endotoxin in the blood of patients with sepsis due to gram-negative bacteria. *N. Engl. J. Med.* **283**, 1313–1316
58. Opal, S. M., Scannon, P. J., Vincent, J. L., White, M., Carroll, S. F., Palardy, J. E., et al. (1999) Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. *J. Infect. Dis.* **180**, 1584–1589
59. Moller, A. S., Bjerre, A., Brusletto, B., Joo, G. B., Brandtzaeg, P., and Kierulf, P. (2005) Chemokine patterns in meningococcal disease. *J. Infect. Dis.* **191**, 768–775
60. Ovstebo, R., Brandtzaeg, P., Brusletto, B., Haug, K. B., Lande, K., Hoiby, E. A., et al. (2004) Use of robotized DNA isolation and real-time PCR to quantify and identify close correlation between levels of *Neisseria meningitidis* DNA and lipopolysaccharides in plasma and cerebrospinal fluid from patients with systemic meningococcal disease. *J. Clin. Microbiol.* **42**, 2980–2987
61. Cabie, A., Farkas, J. C., Fitting, C., Laurian, C., Cormier, J. M., Carlet, J., et al. (1993) High levels of portal TNF-alpha during abdominal aortic surgery in man. *Cytokine* **5**, 448–453
62. Adrie, C., Adib-Conquy, M., Laurent, I., Monchi, M., Vinsonneau, C., Fitting, C., et al. (2002) Successful cardiopulmonary resuscitation after cardiac arrest as a “sepsis-like” syndrome. *Circulation* **106**, 562–568
63. Roumen, R. M., Hendriks, T., Wevers, R. A., and Goris, J. A. (1993) Intestinal permeability after severe trauma and hemorrhagic shock is increased without relation to septic complications. *Arch. Surg.* **128**, 453–457
64. Chancharoenthana, W., Leelahavanichkul, A., Ariyanon, W., Vadcharavivad, S., Phatcharophaswattanakul, S., Kamolratanakul, S., et al. (2021) Leaky gut syndrome is associated with endotoxemia and serum (1->3)-beta-D-glucan in severe dengue infection. *Microorganisms* **9**, 2390
65. Cavaillon, J. M. (2018) Historical links between toxicology and immunology. *Pathog. Dis.* <https://doi.org/10.1093/femspd/fty019>
66. Davies, B., and Cohen, J. (2011) Endotoxin removal devices for the treatment of sepsis and septic shock. *Lancet Infect. Dis.* **11**, 65–71
67. Dellinger, R. P., Bagshaw, S. M., Antonelli, M., Foster, D. M., Klein, D. J., Marshall, J. C., et al. (2018) Effect of targeted polymyxin B hemoperfusion on 28-day mortality in patients with septic shock and elevated endotoxin level: the EUPHRATES randomized clinical trial. *JAMA* **320**, 1455–1463
68. Osawa, I., Goto, T., Kudo, D., Hayakawa, M., Yamakawa, K., Kushimoto, S., et al. (2023) Targeted therapy using polymyxin B hemadsorption in patients with sepsis: a post-hoc analysis of the JSEPTIC-DIC study and the EUPHRATES Trial. *Crit. Care* **27**, 245
69. Cavaillon, J. M. (2013) Sir Marc Armand ruffer and Giulio Bizzozero: the first reports on efferocytosis. *J. Leukoc. Biol.* **93**, 39–43
70. Cavaillon, J. M. (2022) From bacterial poisons to toxins: the early works of pasteurians. *Toxins (Basel)* **14**, 759
71. Baker, M. (2016) Reproducibility: respect your cells! *Nature* **537**, 433–435
72. Medzhitov, R., Preston-Hurlburt, P., and Janeway, C. A., Jr. (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* **388**, 394–397
73. Horng, T., Barton, G. M., Flavell, R. A., and Medzhitov, R. (2002) The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* **420**, 329–333
74. Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat. Immunol.* **9**, 361–368

75. Kawai, T., and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11**, 373–384
76. Hailman, E., Vasselon, T., Kelley, M., Busse, L. A., Hu, M. C., Lichtenstein, H. S., *et al.* (1996) Stimulation of macrophages and neutrophils by complexes of lipopolysaccharide and soluble CD14. *J. Immunol.* **156**, 4384–4390
77. Ryu, J. K., Kim, S. J., Rah, S. H., Kang, J. I., Jung, H. E., Lee, D., *et al.* (2017) Reconstruction of LPS transfer cascade reveals structural determinants within LBP, CD14, and TLR4-MD2 for efficient LPS recognition and transfer. *Immunity* **46**, 38–50
78. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., *et al.* (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med.* **189**, 1777–1782
79. Maeshima, N., and Fernandez, R. C. (2013) Recognition of lipid A variants by the TLR4-MD-2 receptor complex. *Front. Cell. Infect. Microbiol.* **3**, 3
80. Tynan, G. A., McNaughton, A., Jarnicki, A., Tsuji, T., and Lavelle, E. C. (2012) Polymyxin B inadequately quenches the effects of contaminating lipopolysaccharide on murine dendritic cells. *PLoS One* **7**, e37261
81. Jungi, T. W., Miserez, R., Brcic, M., and Pfister, H. (1994) Change in sensitivity to lipopolysaccharide during the differentiation of human monocytes to macrophages *in vitro*. *Experientia* **50**, 110–114
82. Jungi, T. W., Brcic, M., and Eperon, S. (1996) Human macrophages respond to LPS in a serum-independent, CD14-dependent manner. *Immunol. Lett.* **54**, 37–43
83. Xu, D. X., Wang, H., Zhao, L., Ning, H., Chen, Y. H., and Zhang, C. (2007) Effects of low-dose lipopolysaccharide (LPS) pretreatment on LPS-induced intra-uterine fetal death and preterm labor. *Toxicology* **234**, 167–175
84. Lambeth, J. D. (2004) NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol.* **4**, 181–189
85. Haase, R., Kirschning, C. J., Sing, A., Schrottner, P., Fukase, K., Kusumoto, S., *et al.* (2003) A dominant role of Toll-like receptor 4 in the signaling of apoptosis in bacteria-faced macrophages. *J. Immunol.* **171**, 4294–4303
86. Ruckdeschel, K., Pfaffinger, G., Haase, R., Sing, A., Weighardt, H., Hacker, G., *et al.* (2004) Signaling of apoptosis through TLRs critically involves toll/IL-1 receptor domain-containing adapter inducing IFN-beta, but not MyD88, in bacteria-infected murine macrophages. *J. Immunol.* **173**, 3320–3328
87. Huang, X., Feng, Y., Xiong, G., Whyte, S., Duan, J., Yang, Y., *et al.* (2019) Caspase-11, a specific sensor for intracellular lipopolysaccharide recognition, mediates the non-canonical inflammatory pathway of pyroptosis. *Cell Biosci.* **9**, 31
88. Kayagaki, N., Wong, M. T., Stowe, I. B., Ramani, S. R., Gonzalez, L. C., Akashi-Takamura, S., *et al.* (2013) Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* **341**, 1246–1249
89. Shi, J., Zhao, Y., Wang, Y., Gao, W., Ding, J., Li, P., *et al.* (2014) Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* **514**, 187–192
90. Skirecki, T., and Cavaillon, J. M. (2019) Inner sensors of endotoxin - implications for sepsis research and therapy FEMS. *Microbiol. Rev.* **43**, 239–256
91. D'Souza, C. A., and Heitman, J. (2001) Dismantling the *Cryptococcus* coat. *Trends Microbiol.* **9**, 112–113
92. Adrie, C., Monchi, M., Fulgencio, J. P., Cottias, P., Haouache, H., Alvarez-Gonzalez, A., *et al.* (2010) Immune status and apoptosis activation during brain death. *Shock* **33**, 353–362
93. Maisonneuve, C., Bertholet, S., Philpott, D. J., and De Gregorio, E. (2014) Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 12294–12299
94. Bowen, W. S., Gandhapudi, S. K., Kolb, J. P., and Mitchell, T. C. (2013) Immunopharmacology of lipid A mimetics. *Adv. Pharmacol.* **66**, 81–128
95. Erridge, C. (2010) Endogenous ligands of TLR2 and TLR4: agonists or assistants? *J. Leukoc. Biol.* **87**, 989–999
96. Sansonetti, P. J., Phalipon, A., Arondel, J., Thirumalai, K., Banerjee, S., Akira, S., *et al.* (2000) Caspase-1 activation of IL-1beta and IL-18 are essential for *Shigella flexneri*-induced inflammation. *Immunity* **12**, 581–590
97. Cavaillon, J. M., and Haeflner-Cavaillon, N. (1986) Polymyxin-B inhibition of LPS-induced interleukin-1 secretion by human monocytes is dependent upon the LPS origin. *Mol. Immunol.* **23**, 965–969
98. Cavaillon, J. M. (2011) Polymyxin B for endotoxin removal in sepsis. *Lancet Infect. Dis.* **11**, 426–427
99. Domingues, M. M., Inacio, R. G., Raimundo, J. M., Martins, M., Castanho, M. A., and Santos, N. C. (2012) Biophysical characterization of polymyxin B interaction with LPS aggregates and membrane model systems. *Biopolymers* **98**, 338–344
100. Jaber, B. L., Sundaram, S., Cendoroglo Neto, M., King, A. J., and Pereira, B. J. (1998) Polymyxin-B stimulates tumor necrosis factor-alpha production by human peripheral blood mononuclear cells. *Int. J. Artif. Organs* **21**, 269–273
101. Damais, C., Jupin, C., Parant, M., and Chedid, L. (1987) Induction of human interleukin-1 production by polymyxin B. *J. Immunol. Methods* **101**, 51–56
102. Laude-Sharp, M., Haeflner-Cavaillon, N., Caroff, M., Lantreibecq, F., Pusineri, C., and Kazatchkine, M. D. (1990) Dissociation between the interleukin 1-inducing capacity and Limulus reactivity of lipopolysaccharides from gram-negative bacteria. *Cytokine* **2**, 253–258
103. Mancek-Keber, M., and Jerala, R. (2015) Postulates for validating TLR4 agonists. *Eur. J. Immunol.* **45**, 356–370
104. Mack, L., Brill, B., Delis, N., and Groner, B. (2014) Endotoxin depletion of recombinant protein preparations through their preferential binding to histidine tags. *Anal. Biochem.* **466**, 83–88
105. Hodes, D. S., Heon, D., Hass, A., Hyatt, A. C., and Hodes, H. L. (1987) Reaction of fungal products with amebocyte lysates of the Japanese horseshoe crab, *Tachypleus tridentatus*. *J. Clin. Microbiol.* **25**, 1701–1704
106. Tsan, M. F., and Gao, B. (2007) Pathogen-associated molecular pattern contamination as putative endogenous ligands of Toll-like receptors. *J. Endotoxin Res.* **13**, 6–14
107. Hirata, N., Yanagawa, Y., Ebihara, T., Seya, T., Uematsu, S., Akira, S., *et al.* (2008) Selective synergy in anti-inflammatory cytokine production upon cooperated signaling *via* TLR4 and TLR2 in murine conventional dendritic cells. *Mol. Immunol.* **45**, 2734–2742
108. Liang, M. D., Bagchi, A., Warren, H. S., Tehan, M. M., Trigilio, J. A., Beasley-Topliffe, L. K., *et al.* (2005) Bacterial peptidoglycan-associated lipoprotein: a naturally occurring toll-like receptor 2 agonist that is shed into serum and has synergy with lipopolysaccharide. *J. Infect. Dis.* **191**, 939–948
109. Fritz, J. H., Girardin, S. E., Fitting, C., Werts, C., Mengin-Lecreulx, D., Caroff, M., *et al.* (2005) Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur. J. Immunol.* **35**, 2459–2470
110. Siddiqui, M. A., and Yamashita, M. (2021) Toll-like receptor (TLR) signaling enables cyclic GMP-AMP synthase (cGAS) sensing of HIV-1 infection in macrophages. *mBio* **12**, e0281721
111. Tirsoaga, A., Novikov, A., Adib-Conquy, M., Werts, C., Fitting, C., Cavaillon, J. M., *et al.* (2007) Simple method for repurification of endotoxins for biological use. *Appl. Environ. Microbiol.* **73**, 1803–1808
112. Yu, L., Wang, L., and Chen, S. (2010) Endogenous toll-like receptor ligands and their biological significance. *J. Cell. Mol. Med.* **14**, 2592–2603
113. Ohto, U., Fukase, K., Miyake, K., and Shimizu, T. (2012) Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 7421–7426
114. Park, B. S., Song, D. H., Kim, H. M., Choi, B. S., Lee, H., and Lee, J. O. (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* **458**, 1191–1195
115. Wang, Y., Su, L., Morin, M. D., Jones, B. T., Whitby, L. R., Surakattula, M. M., *et al.* (2016) TLR4/MD-2 activation by a synthetic agonist with no similarity to LPS. *Proc. Natl. Acad. Sci. U. S. A.* **113**, E884–E893
116. Michaeli, A., Mezan, S., Kuhbacher, A., Finkelmeier, D., Elias, M., Zatschin, M., *et al.* (2018) Computationally designed Bispecific MD2/CD14

- binding peptides show TLR4 agonist activity. *J. Immunol.* **201**, 3383–3391
117. Su, L., Athamna, M., Wang, Y., Wang, J., Freudenberg, M., Yue, T., *et al.* (2021) Sulfatides are endogenous ligands for the TLR4-MD-2 complex. *Proc. Natl. Acad. Sci. U. S. A.* **118**, e2105316118
 118. Wallin, R. P., Lundqvist, A., More, S. H., von Bonin, A., Kiessling, R., and Ljunggren, H. G. (2002) Heat-shock proteins as activators of the innate immune system. *Trends Immunol.* **23**, 130–135
 119. Marinček, B. C., Kuhnle, M. C., Srokowski, C., Schild, H., Hammerling, G., and Momburg, F. (2008) Heat shock protein-antigen fusions lose their enhanced immunostimulatory capacity after endotoxin depletion. *Mol. Immunol.* **46**, 181–191
 120. Bausinger, H., Lipsker, D., Ziyilan, U., Manie, S., Briand, J. P., Cazenave, J. P., *et al.* (2002) Endotoxin-free heat-shock protein 70 fails to induce APC activation. *Eur. J. Immunol.* **32**, 3708–3713
 121. Zheng, H., Nagaraja, G. M., Kaur, P., Asea, E. E., and Asea, A. (2010) Chaperone function of recombinant Hsp72 produced in insect cells using a baculovirus expression system is retained. *J. Biol. Chem.* **285**, 349–356
 122. Murshid, A., Borges, T. J., Bonorino, C., Lang, B. J., and Calderwood, S. K. (2019) Immunological outcomes mediated upon binding of heat shock proteins to scavenger receptors SCARF1 and LOX-1, and endocytosis by mononuclear phagocytes. *Front. Immunol.* **10**, 3035
 123. Stefano, L., Racchetti, G., Bianco, F., Passini, N., Gupta, R. S., Panina Bordignon, P., *et al.* (2009) The surface-exposed chaperone, Hsp60, is an agonist of the microglial TREM2 receptor. *J. Neurochem.* **110**, 284–294
 124. Ho, J. D., Takara, L. E. M., Monaris, D., Goncalves, A. P., Souza-Filho, A. F., de Souza, G. O., *et al.* (2021) GroEL protein of the *Leptospira* spp. interacts with host proteins and induces cytokines secretion on macrophages. *BMC Microbiol.* **21**, 99
 125. Cavillon, J. M., and Osuchowski, M. F. (2021) COVID-19 and earlier pandemics, sepsis, and vaccines: a historical perspective. *J. Intensive Med.* **1**, 4–13
 126. Zhao, Y., Kuang, M., Li, J., Zhu, L., Jia, Z., Guo, X., *et al.* (2021) SARS-CoV-2 spike protein interacts with and activates TLR41. *Cell Res.* **31**, 818–820
 127. Shirato, K., and Kizaki, T. (2021) SARS-CoV-2 spike protein S1 subunit induces pro-inflammatory responses via toll-like receptor 4 signaling in murine and human macrophages. *Heliyon* **7**, e06187
 128. Khan, S., Shafiei, M. S., Longoria, C., Schoggins, J. W., Savani, R. C., and Zaki, H. (2021) SARS-CoV-2 spike protein induces inflammation via TLR2-dependent activation of the NF-kappaB pathway. *Elife* **10**, e68563
 129. Corral-Vazquez, C., Aguilar-Quesada, R., Catalina, P., Lucena-Aguilar, G., Ligerio, G., Miranda, B., *et al.* (2017) Cell lines authentication and mycoplasma detection as minimum quality control of cell lines in biobanking. *Cell Tissue Bank.* **18**, 271–280
 130. Zheng, M., Karki, R., Williams, E. P., Yang, D., Fitzpatrick, E., Vogel, P., *et al.* (2021) TLR2 senses the SARS-CoV-2 envelope protein to produce inflammatory cytokines. *Nat. Immunol.* **22**, 829–838
 131. Cinquegrani, G., Spigoni, V., Iannozzi, N. T., Parello, V., Bonadonna, R. C., and Dei Cas, A. (2022) SARS-CoV-2 Spike protein is not pro-inflammatory in human primary macrophages: endotoxin contamination and lack of protein glycosylation as possible confounders. *Cell Biol. Toxicol.* **38**, 667–678
 132. Petruk, G., Puthia, M., Petrlova, J., Samsudin, F., Stromdahl, A. C., Cerps, S., *et al.* (2020) SARS-CoV-2 spike protein binds to bacterial lipopolysaccharide and boosts proinflammatory activity. *J. Mol. Cell. Biol.* **12**, 916–932
 133. Choudhury, A., and Mukherjee, S. (2020) In silico studies on the comparative characterization of the interactions of SARS-CoV-2 spike glycoprotein with ACE-2 receptor homologs and human TLRs. *J. Med. Virol.* **92**, 2105–2113
 134. Bhattacharya, M., Sharma, A. R., Mallick, B., Sharma, G., Lee, S. S., and Chakraborty, C. (2020) Immunoinformatics approach to understand molecular interaction between multi-epitopic regions of SARS-CoV-2 spike-protein with TLR4/MD-2 complex. *Infect. Genet. Evol.* **85**, 104587
 135. Chen, L. C., Lei, H. Y., Liu, C. C., Shiesh, S. C., Chen, S. H., Liu, H. S., *et al.* (2006) Correlation of serum levels of macrophage migration inhibitory factor with disease severity and clinical outcome in dengue patients. *Am. J. Trop. Med. Hyg.* **74**, 142–147
 136. Lee, M. F., Voon, G. Z., Lim, H. X., Chua, M. L., and Poh, C. L. (2022) Innate and adaptive immune evasion by dengue virus. *Front. Cell. Infect. Microbiol.* **12**, 1004608
 137. Beatty, P. R., Puerta-Guardo, H., Killingbeck, S. S., Glasner, D. R., Hopkins, K., and Harris, E. (2015) Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. *Sci. Transl. Med.* **7**, 304ra141
 138. Chao, C. H., Wu, W. C., Lai, Y. C., Tsai, P. J., Perng, G. C., Lin, Y. S., *et al.* (2019) Dengue virus nonstructural protein 1 activates platelets via Toll-like receptor 4, leading to thrombocytopenia and hemorrhage. *PLoS Pathog.* **15**, e1007625
 139. Modhiran, N., Watterson, D., Muller, D. A., Panetta, A. K., Sester, D. P., Liu, L., *et al.* (2015) Dengue virus NS1 protein activates cells via Toll-like receptor 4 and disrupts endothelial cell monolayer integrity. *Sci. Transl. Med.* **7**, 304ra142
 140. Chen, J., Ng, M. M., and Chu, J. J. (2015) Activation of TLR2 and TLR6 by dengue NS1 protein and its implications in the immunopathogenesis of dengue virus infection. *PLoS Pathog.* **11**, e1005053
 141. Modhiran, N., Watterson, D., Blumenthal, A., Baxter, A. G., Young, P. R., and Stacey, K. J. (2017) Dengue virus NS1 protein activates immune cells via TLR4 but not TLR2 or TLR6. *Immunol. Cell Biol.* **95**, 491–495
 142. Hoshino, S., Konishi, M., Mori, M., Shimura, M., Nishitani, C., Kuroki, Y., *et al.* (2010) HIV-1 Vpr induces TLR4/MyD88-mediated IL-6 production and reactivates viral production from latency. *J. Leukoc. Biol.* **87**, 1133–1143
 143. Nazli, A., Kafka, J. K., Ferreira, V. H., Anipindi, V., Mueller, K., Osborne, B. J., *et al.* (2013) HIV-1 gp120 induces TLR2- and TLR4-mediated innate immune activation in human female genital epithelium. *J. Immunol.* **191**, 4246–4258
 144. Akbarshahi, H., Axelsson, J. B., Said, K., Malmstrom, A., Fischer, H., and Andersson, R. (2011) TLR4 dependent heparan sulphate-induced pancreatic inflammatory response is IRF3-mediated. *J. Transl. Med.* **9**, 219
 145. Ben Hajj, N., Leghmari, K., Planes, R., Thieblemont, N., and Bahraoui, E. (2013) HIV-1 Tat protein binds to TLR4-MD2 and signals to induce TNF-alpha and IL-10. *Retrovirology* **10**, 123
 146. Ouyang, J., Yan, J., Zhou, X., Isnard, S., Harypursat, V., Cui, H., *et al.* (2023) Relevance of biomarkers indicating gut damage and microbial translocation in people living with HIV. *Front. Immunol.* **14**, 1173956
 147. Ancuta, P., Kamat, A., Kunstman, K. J., Kim, E. Y., Autissier, P., Wurcel, A., *et al.* (2008) Microbial translocation is associated with increased monocyte activation and dementia in AIDS patients. *PLoS One* **3**, e2516
 148. Bonhomme, D., and Werts, C. (2022) Host and species specificities of pattern recognition receptors upon infection with *Leptospira interrogans*. *Front. Cell. Infect. Microbiol.* **12**, 932137
 149. Santecchia, L., Bonhomme, D., Papadopoulos, S., Escoll, P., Giraud-Gatineau, A., Moya-Nilges, M., *et al.* (2022) Alive pathogenic and Saprophytic leptospires enter and exit human and mouse macrophages with no intracellular replication. *Front. Cell. Infect. Microbiol.* **12**, 936931
 150. Bonhomme, D., Hernandez-Trejo, V., Papadopoulos, S., Pigache, R., Fanton d'Andon, M., Outlioua, A., *et al.* (2023) *Leptospira interrogans* prevents macrophage cell death and pyroptotic IL-1beta release through its atypical lipopolysaccharide. *J. Immunol.* **210**, 459–474
 151. Che, R., Ding, S., Zhang, Q., Yang, W., Yan, J., and Lin, X. (2019) Haemolysin Sph2 of *Leptospira interrogans* induces cell apoptosis via intracellular reactive oxygen species elevation and mitochondrial membrane injury. *Cell. Microbiol.* **21**, e12959
 152. Faisal, S. M., Varma, V. P., Subathra, M., Azam, S., Sunkara, A. K., Akif, M., *et al.* (2016) *Leptospira* surface adhesin (Lsa21) induces Toll like receptor 2 and 4 mediated inflammatory responses in macrophages. *Sci. Rep.* **6**, 39530
 153. Fang, J. Q., Imran, M., Hu, W. L., Ojcius, D. M., Li, Y., Ge, Y. M., *et al.* (2018) vWA proteins of *Leptospira interrogans* induce hemorrhage in leptospirosis by competitive inhibition of vWF/GPIIb-mediated platelet aggregation. *EBioMedicine* **37**, 428–441

154. Hsu, S. H., Chu, C. H., Tian, Y. C., Chang, M. Y., Chou, L. F., Sun, Y. J., *et al.* (2020) Crystal structure of *Leptospira* leucine-rich repeat 20 reveals a novel E-cadherin binding protein to induce NGAL expression in HK2 cells. *Biochem. J.* **477**, 4313–4326
155. Kumar, A., Varma, V. P., Sridhar, K., Abdullah, M., Vyas, P., Ashiq Thalappil, M., *et al.* (2021) Deciphering the role of *Leptospira* surface protein LigA in modulating the host innate immune response. *Front. Immunol.* **12**, 807775
156. Hsu, S. H., Chang, M. Y., Lin, S. M., Ko, Y. C., Chou, L. F., Tian, Y. C., *et al.* (2021) Peptidoglycan mediates *Leptospira* outer membrane protein Loa22 to toll-like receptor 2 for inflammatory interaction: a novel innate immune recognition. *Sci. Rep.* **11**, 1064
157. Passalia, F. J., Heinemann, M. B., de Andrade, S. A., Nascimento, A., and Vieira, M. L. (2020) *Leptospira interrogans* Bat proteins impair host hemostasis by fibrinogen cleavage and platelet aggregation inhibition. *Med. Microbiol. Immunol.* **209**, 201–213
158. Wang, H., Wu, Y., Ojcius, D. M., Yang, X. F., Zhang, C., Ding, S., *et al.* (2012) Leptospiral hemolysins induce proinflammatory cytokines through Toll-like receptor 2-and 4-mediated JNK and NF-kappaB signaling pathways. *PLoS One* **7**, e42266
159. Kumar, A., Varma, V. P., and Faisal, S. M. (2022) Screening of surface-exposed lipoproteins of *Leptospira* involved in modulation of host innate immune response. *Front. Microbiol.* **13**, 761670
160. Mamat, U., Wilke, K., Bramhill, D., Schromm, A. B., Lindner, B., Kohl, T. A., *et al.* (2015) Detoxifying *Escherichia coli* for endotoxin-free production of recombinant proteins. *Microb. Cell Fact.* **14**, 57
161. Muroi, M., and Tanamoto, K. (2006) Structural regions of MD-2 that determine the agonist-antagonist activity of lipid IVa. *J. Biol. Chem.* **281**, 5484–5491
162. Lozano-Aponte, J., Scior, T., Ambrosio, F. N. M., Gonzalez-Melchor, M., and Alexander, C. (2020) Exploring electrostatic patterns of human, murine, equine and canine TLR4/MD-2 receptors. *Innate Immun.* **26**, 364–380
163. Souza, C. C., Guimaraes, J. M., Pereira, S. D. S., and Mariuba, L. A. M. (2021) The multifunctionality of expression systems in *Bacillus subtilis*: emerging devices for the production of recombinant proteins. *Exp. Biol. Med. (Maywood)* **246**, 2443–2453
164. Shahar, E., Emquies, K., Bloch, I., Eliahu, D., Ben Adiva, R., Pitcovski, J., *et al.* (2023) Endotoxin-free gram-negative bacterium as a system for production and secretion of recombinant proteins. *Appl. Microbiol. Biotechnol.* **107**, 287–298
165. Strasser, R. (2023) Plant glycoengineering for designing next-generation vaccines and therapeutic proteins. *Biotechnol. Adv.* **67**, 108197
166. Fujiwara, Y., Yang, L., Takaiwa, F., and Sekikawa, K. (2016) Expression and purification of recombinant mouse interleukin-4 and -6 from transgenic rice seeds. *Mol. Biotechnol.* **58**, 223–231
167. Liu, C., Gong, J. S., Su, C., Li, H., Li, H., Rao, Z. M., *et al.* (2022) Pathway engineering facilitates efficient protein expression in *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* **106**, 5893–5912
168. Borriello, F., Poli, V., Shrock, E., Spreafico, R., Liu, X., Pishesha, N., *et al.* (2022) An adjuvant strategy enabled by modulation of the physical properties of microbial ligands expands antigen immunogenicity. *Cell* **185**, 614–629.e21
169. Willcocks, S., Offord, V., Seyfert, H. M., Coffey, T. J., and Werling, D. (2013) Species-specific PAMP recognition by TLR2 and evidence for species-restricted interaction with Dectin-1. *J. Leukoc. Biol.* **94**, 449–458