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# On the translocation of bacteria and their lipopolysaccharides between blood and peripheral locations in chronic, inflammatory diseases: the central roles of LPS and LPS-induced cell death?

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We have recently highlighted (and added to) the considerable evidence that blood can contain dormant bacteria. By definition, such bacteria may be resuscitated (and thus proliferate). This may occur under conditions that lead to or exacerbate chronic, inflammatory diseases that are normally considered to lack a microbial component. Bacterial cell wall components, such as the endotoxin lipopolysaccharide (LPS) of Gram-negative strains, are well known as potent inflammatory agents, but should normally be cleared. Thus, their continuing production and replenishment from dormant bacterial reservoirs provides an easy explanation for the continuing, low-grade inflammation (and inflammatory cytokine production) that is characteristic of many such diseases. Although experimental conditions and determinants have varied considerably between investigators, we summarise the evidence that in a great many circumstances LPS can play a central role in all of these processes, including in particular cell death processes that permit translocation between the gut, blood and other tissues. Such localised cell death processes might also contribute strongly to the specific diseases of interest. The bacterial requirement for free iron explains the strong co-existence in these diseases of iron dysregulation, LPS production. and inflammation. Overall this analysis provides an integrative picture, with significant predictive power, that is able to link these processes via the centrality of a dormant blood microbiome that can resuscitate and shed cell wall components.

### Insight, innovation, integration

The Biological Insight of this manuscript is that while dormant bacteria, including those in blood, are normally unnoticed (as they are invisible to conventional methods of culture), they can by definition be resuscitated and then proliferate for at least a certain number of generations before possibly returning to a state of dormancy. This allows a continuing production and shedding of potent inflammatory agents such as the lipopolysaccharide (LPS) characteristic of the Gram-negative cell wall. Well-established pathways link LPS (sensu lato) to inflammatory cytokine production, and to cell death via apoptosis, programmed necrosis, and pyroptosis, with the accompanying microparticle formation known to occur with these cell death mechanisms. Cytokine-mediated cell death mechanisms that permit both (i) the translocation of bacteria between blood and other tissues, and (ii) localised proliferation leading to inflammation and cell death, are likely to be a major component of the various disease manifestations involved. One established requirement for bacterial resuscitation and proliferation comes from the need for available iron. The Technological Innovation is the use of advanced microscopy techniques to detect these dormant bacteria as well as microparticle formation. The Benefit of Integration comes (i) from bringing together these multiple biochemical elements (bacterial dormancy and resuscitation, LPS-induced inflammatory cytokine production, cytokine-induced cell death, cell-death-induced translocation, and localised cell death induced by LPS), and (ii) by showing their commonality, and the centrality of LPS, across a range of chronic, inflammatory diseases normally considered to lack a microbial component.

### Introduction

Blood is normally considered a sterile environment in the sense of lacking active microbes, since any bacteraemia or sepsis is potentially extremely life-threatening. However, this does not exclude the presence in blood of dormant bacteria, that by definition<sup>2,3</sup> are not growing but resist detection by standard culture techniques, yet are not 'dead' as they may be resuscitated

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and then proliferate. We have recently summarised the considerable evidence<sup>4,5</sup> to the effect that human blood contains an authentic but dormant microbiome that can contribute significantly to a large variety of chronic inflammatory diseases, a set of diseases that is strikingly similar to those for which we had previously noted the presence of iron dysregulation<sup>6–10</sup> and hypercoagulability.<sup>9</sup>

Given the well-established facts (i) that microbial growth in vivo is normally strongly limited by the (non-) availability of free iron (e.g. ref. 11-21), and (ii) that bacterial components such as lipopolysaccharide (LPS) are strongly inflammatory (e.g. ref. 22 and 23), such an analysis leads to the recognition that the iron-related inflammatory diseases also have a major microbial component involving the resuscitation of dormant organisms and their shedding of inflammatory molecules, and especially of cell wall components such as LPS (Fig. 1). LPS is commonly known as endotoxin, albeit that it is frequently shed, and we shall use this name interchangeably unless otherwise specified. Most work has been done with LPS from Gramnegative bacteria, but unless specified, we recognise that much of what we have to say should be taken to apply to inflammatory processes catalysed by cell wall components (such as lipoteichoic acids<sup>24</sup>) from Gram-positive organisms, ultramicrobacteria, <sup>25</sup> and potentially (though there seems to be relatively little work on this<sup>26-29</sup>) from the cell envelopes of archaea. Also, though many of the ideas developed here very likely apply to them too, and there is a considerable literature, we shall not discuss viruses,<sup>30</sup> nor mycoplasmas<sup>31–33</sup> in much detail.

The earlier overviews<sup>4,5</sup> recognised that the chief sources of the blood microbiome were likely to be *via* translocations of microbes from the gut and oral cavities, and although a number of the diseases discussed were neurodegenerative in nature, we did not look at the evidence (and mechanism) for the transport of cells from blood into tissues such as the CNS. A chief purpose of the present review is thus to take a systems

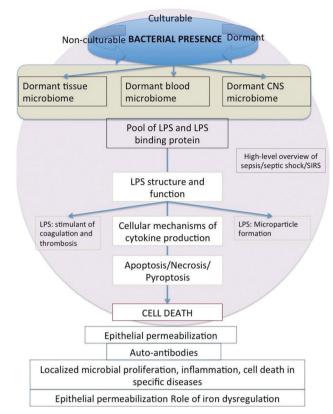


Fig. 1 An overall illustration of the headline processes involved in chronic inflammation and disease aetiology mediated *via* the resuscitation of dormant microbes and the increased production and shedding of cell wall components.

approach, designed to bring together the evidence for the strong involvement of microbes and their inflammatory bacterial cell wall components in both the diseases themselves and their dynamics, and relating the known ability of LPS and related



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Integrative Biology Perspective

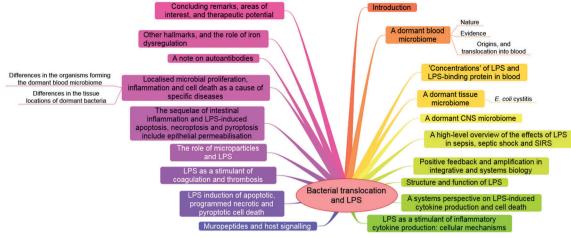


Fig. 2 A 'mind map'<sup>34</sup> summarising the article.

component to induce (mainly apoptotic) cell death. This turns out to be sufficient to explain many of the acute and chronic sequelae of the presence of microbes and their cell envelope products in mammals. An overview of the present article is given in the form of a mind map (Fig. 2). As background, we first discuss microbial dormancy, culturability and non-culturability.

### A dormant blood microbiome

As foreshadowed in the introduction, we<sup>4,5</sup> and others<sup>35,36</sup> have summarised the rather extensive evidence that the presence of bacteria in blood is - perhaps unsurprisingly, given the assumption that blood is normally sterile - much commoner than is usually supposed, and we have pointed out<sup>4,5</sup> that, in the usual absence of overt bacteraemia, such organisms are best considered as being in a dormant physiological state.

Dormancy is defined as a reversible non-replicating state, often of low metabolic activity. Leaving aside obviously specialised forms such as spores and seeds ('constitutive dormancy'<sup>2</sup>), in non-sporulating bacteria it manifests typically as an inability of an individual cell to proliferate (e.g. to form a colony) under conditions normally considered adequate for cultivation, but where the cell is not operationally 'dead'<sup>3</sup> in that it can revert to a state of 'aliveness' or 'culturability' via processes referred to as resuscitation. Thus, by definition, dormant cells are resuscitable, but this necessarily operational definition means that we can only tell that they were dormant, not that they are dormant. 2,3 Indeed, the ability to undergo dormancy (as with pheromone production<sup>37</sup>) is increasingly being recognised as an adaptive phenotypic trait (e.g. ref. 38-41). Ewald in particular (e.g. ref. 42-44) has stressed the evolutionary aspects of infectious diseases.

### **Evidence**

The evidence for a dormant blood microbiome comes mainly<sup>4,5</sup> from its direct assessment using culture-independent methods (although we know of Domingue's resuscitation papers<sup>45–47</sup>). Culture-independent methods include the detection of blood

(or tissue) microbial macromolecules such as rDNA48-59 and the direct visualisation of cells using ultramicroscopic methods (e.g. ref. 5, 43, 45, 46, 60-64). In particular, it is recognised that dormant blood bacteria could 'hide' (or at least survive 65-68) not only in white cells (e.g. ref. 69) but also within the (more than 1000-fold more numerous) erythrocytes. The significance of this, of course, is the sheer numbers that may be involved. If only one in 50 000 erythrocytes (that are present in blood at ca.  $5 \times 10^9 \,\mathrm{mL}^{-1}$ ) each harboured just a single dormant bacterium, there would still be more than  $10^5$  mL<sup>-1</sup> (a number equivalent in urine to the threshold normally given for culturable cells in defining clinical bacteriuria).

Although there is evidence that a surprisingly large variety of bacteria can invade erythrocytes,5 we know next to nothing about how they enter and egress from such cells. Even whether the latter involves pore-forming toxins<sup>70,71</sup> or haemolysins<sup>72</sup> that may effect membrane destruction, is unclear.

### Origins, and translocation into blood

We also rehearsed<sup>5</sup> the considerable evidence that minor leakages from the gut microbiome (e.g. ref. 73-75), even if only a tiny fraction of the 10-100 trillion <sup>76</sup> cells involved, whether *via* specialised structures such as M cells or more significant breaches in the gut epithelium (as can also occur in some cancers<sup>77</sup> and stroke<sup>78</sup>), are more than sufficient to provide a continuing inoculum to the bloodstream. Clearly the innate and adaptive immune system will normally remove those organisms threatening growth and noticeable bacteraemia, but this statement does not account for the fraction that become dormant and hide therefrom (whether geographically in cells and/or by losing their immunogenic potential, for instance by creating L-forms<sup>64</sup>). While the dormant bacteria do seem mainly to be hidden inside cells, their inflammatory products may not be. In the blood, LPS is typically bound either to an LPS binding protein (LBP)<sup>79-82</sup> which is a glycoprotein with a molecular weight of some 58 kDa<sup>83</sup> (452 amino acids<sup>84</sup>) or to the lipoprotein ApoE that is protective against LPS.85-87 The ApoE4 polymorphism is of course well known as a genetic

locus favouring the development of Alzheimer's disease (e.g. ref. 87–92).

## 'Concentrations' of LPS and LPS-binding protein in blood

Our central argument is that low grade inflammation is mainly effected via the continuing production and shedding of LPS (and similar molecules) as dormant bacteria periodically awaken, proliferate and produce LPS before returning to dormancy. If this is going to be true, it is instructive to determine how much LPS and related molecules are typically found in human blood under various conditions. The potential load of LPS in the alimentary canal is  $\sim 1$  g.<sup>93</sup> We note, of course, that (as with serum ferritin8) the basis of these assays used to estimate concentration is typically a binding reaction, whether to an antibody or (in the case of LPS) based on a Limulus amoebocyte lysate (or its recombinant factor C94). Thus these and other (e.g. ref. 95-97) assays typically measure the (thermodynamically active) free forms, while the total amounts may be very much greater if (as with LPS) they are mainly bound to LBP or ApoE of HDL/cholesterol, or even monocyte surfaces. 98 Indeed, given that HDL-cholesterol is capable of sequestering LPS99 (and lipoteichoic acid<sup>100</sup>), it is not surprising that there is considerable evidence that HDL-cholesterol is protective against sepsis and sepsis-related death, 83,101-107 showing further the importance of free LPS levels in disease prognosis.

This said, it is important to point out that if a substance is hydrophobic, *i.e.* poorly water-soluble, and its targets are hydrophobic (*i.e.* insoluble) as well, its measured potency also depends on the concentration of the hydrophobic elements containing the target (or otherwise). In such circumstances, it is arguably better to speak of functional concentrations in terms of nmol per nmol target or similar, rather than in concentration terms (*e.g.* nM). In a similar vein, when considering properties such as cell death, what matters is the distribution of ligands between targets and the fraction of cells that die. In other words, if an added toxic molecule kills a cell (*i.e.* irreversibly) then this is a quantised property of the molecule, and again 'concentrations' are not an entirely meaningful manner with which to describe the toxic stimulus. 3,110,111

Our main purpose here, though, is comparative, and aimed at obtaining a feel for the typical concentrations in health and disease, and those that are used in research studies. Note that as well as coming from infections, LPS is a common component of dust. Thus, reported LPS 'concentrations' in healthy subjects seem to be of the order of 10–15 ng L<sup>-1</sup>, while those of LBP are roughly 1 000 000 times greater at 5–15 mg L<sup>-1</sup> (with both values increasing during sepsis) (Tables 1 and 2). LPS challenges of 5–100 µg per patient are commonly administered as experimental challenges and seen as 'safe'. The larger volume, if distributed in 5 L of blood (a typical human value) equates to 20 000 ng L<sup>-1</sup>, which is obviously much higher than those free amounts typically measured even in sepsis. In terms of relating LPS to microbial biomass (see ref. 113),

Table 1 A summary of LBP (LPS-binding protein) concentrations in health and disease

Tissue type	LBP in disease (mg $L^{-1}$ )	LBP in control (mg $L^{-1}$ )	Place	Ref.
Bacterial gastrointestinal infections Crohn's disease (CD) and ulcerative colitis (UC) Diabetes type 2	$28.5 \pm 16.5$ 57.11 (49.4-65.8) $19.78 \pm 6.40$		Serum Plasma Serum	122 123 124
Endocarditis Infectious endocarditis Noninfectious heart valve diseases	Median 33.41 Median 6.67	Median 5.61	Serum	125
Inflammatory bowel disease	52.7 (45.4–64.6)	39.1 (32.1–43.7)	Serum	123
Lifestyle factors				
Smoking Obese Overweight Metabolic syndrome	7.11 (5.85–8.74) 5.90 (5.09–7.67) 5.90 (5.09–7.67) 6.82 (5.48–8.40)	7.18 (5.42–9.15) 7.75 (6.35–9.47) 7.29 (5.96–8.78) 8.02 (6.63–9.82)	Serum Serum Serum Serum Plasma	83
Obesity, T2D and metabolic syndrome	27	10	Piasma	126
Liver Hepatocytes Hepatic macrophages Urinary tract infection in childhood	5 to 15 LPS concentration were 10-fold his >43.23	—gher than in the healthy controls	Cells Plasma Serum	127 128 129
Sepsis Sepsis/septic shock LBP concentration at onset of severe sepsis	46.2 (3.74–155)	7.94	Serum	127
Sepsis in neonates Late-onset neonatal sepsis (LONS) Gram +ve or Gram –ve sepsis (Higher in survivors) Septic shock tests Non-survivors 121 vs. 77 at 48 h Remained much higher in non-survivors	Median 36.6 17.5 216 31 200 116–132 baseline 34–55	Median 7.8 Unstated 16 4 5–15	Plasma Plasma Plasma Plasma Unstated Serum Serum	130 131 132 133 134 135 136

Table 2 A compilation of LPS levels observed in health and disease. An accepted conversion factor between endotoxin units (EU) and ng LPS is 1 ng endotoxin (LPS) = 10 EU

Disease	LPS in disease (ng $L^{-1}$ unless indicated as EU $mL^{-1}$ )	LPS in controls (ng $L^{-1}$ unless indicated as EU $mL^{-1}$ )	Tissue type	Ref.
Healthy individuals Non-obese, post-menopausal women	_	0.15 to 0.35 EU mL <sup>-1</sup> 10-20	Plasma Serum	137 93
Healthy controls		5	Plasma	133
HIV infection Inflammatory bowel disease	60	10.0 (2.0.06.2)	Serum	138 123
Non-alcoholic fatty liver disease	12.6 (5.9–16.2) 7.8–14.8 EU mL <sup>-1</sup>	12.2 (3.8–26.3) 3.2–5.2 EU mL <sup>-1</sup>	Serum	139
Sepsis	200		pl	422
	300 470	7.3 Not noted	Plasma Whole blood	133 140
Type 1 diabetes Microalbuminuria group Normoalbuminuric group	31–60 EU mL <sup>-1</sup> 38–74 EU mL <sup>-1</sup>		Plasma LAL assay Plasma LAL assay	141
Type 2 diabetes Non-obese postmemoposal women Diabetic non-obese postmenoposal women	$-$ 0.39 $\pm$ 0.03 EU mL $^{-1}$	$0.37 \pm 0.02 \; EU \; mL^{-1}$	Plasma LAL assay Plasma LAL assay	93
Insulin-treated diabetes Atherosclerosis	6.6–10.7 EU mL $^{-1}$ Above 50 gave 3× greater chance of atherosclerosis	3.1–5.1 EU mL <sup>-1</sup> 14	Serum	142 143

Watson and colleagues<sup>114</sup> showed in laboratory cultures that LPS amounted to some 50 fg cell<sup>-1</sup> in a logarithmic growth phase, falling to 29 fg cell<sup>-1</sup> in stationary phase, but in the oligotrophic conditions of seawater was just some 2.8 fg cell<sup>-1</sup>. This shows at once that LPS contents per cell can be quite variable, and that bacteria can shed a considerable amount of LPS at no major harm to themselves. On the basis that 1 mg dry weight of bacteria is about 10<sup>9</sup> cells, each cell is about 1 pg, so 50 fg LPS per cell equates to about 5% of its dry weight, a reasonable and self-consistent figure for approximate calculations. To deal with the fact that LPS is typically not a molecularly defined substance, its activity is sometimes reported in 'endotoxin units' (EU) based on a standard taken 115 from an E. coli O55:B5 strain; an approximate relationship is that 1 ng endotoxin  $\sim 10$  EU. While the *Limulus* amoebocyte lysate assay is widely and effectively used as a test for pyrogens in parenteral solutions, its use in the estimation of LPS in blood is not considered especially reliable, 116-120 and it may be better to look more closely at LBP. This said, it is LPS that is the stimulus, and thus knowing its effective concentration is important. Unfortunately (Table 2), although the values in sepsis are considerably greater than are those in controls, there is a rather substantial variation between different studies, likely reflecting the rather different qualities of the assays used, the variation in the nature of the LPS (which is not a molecular entity), and the fact that much of the (rather hydrophobic) LPS in vivo is bound to other substances such that the result of the assay depends in significant measure on the extent and nature of any pre-extraction methods employed. Indeed, there are surprisingly few measurements of LPS in non-infectious lowgrade inflammation, and very little evidence that plasma or serum LPS might be a particularly useful marker of it. The situation is a little clearer with LPS-binding protein (LBP), with a much more obvious distinction between controls and those with sepsis. Less severe instances of infection include a median of 16 mg L<sup>-1</sup> for cirrhosis (interestingly reversed by the antibiotic norfloxacin).<sup>121</sup> Thus on the basis of present assay methods, there seems little benefit of seeking to follow the behaviour of a dormant blood microbiome with LPS measurements.

### A dormant tissue microbiome

Our previous reviews<sup>4,5</sup> (and many other works, e.g. those summarised in ref. 43, 62, 64 and 144) outlined in some detail the fact that many known infectious agents can enter cells and persist intracellularly, and those discussions are not repeated in detail here. Indeed, the very existence of eukaryotes is considered to be based on the intracellular uptake of prokaryotes to form structures such as mitochondria, 145 and there is increasing evidence for dinitrogen fixation by endosymbionts in plant leaf cells (e.g. ref. 146 and 147). Regarding human tissue, as Nash and colleagues put it, 144 "the blood is the most effective vehicle of all for the spread of microbes through the body. After entering the blood they can be transported within a minute or two to a vascular bed in any part of the body. In small vessel such as capillaries and sinusoids where blood flows slowly, there is an opportunity for the microorganism to be arrested and to establish infection in neighbouring tissues." (The same holds true, of course, for circulating tumour cells and their role in metastasis.) Later we shall look at this translocation from blood to tissues in more detail. However, we first mention an example that we did not deal with previously in much detail, viz. E. coli-based cystitis.

### E. coli-based cystitis

Cystitis (inflammation of the bladder) is commonly caused by urinary tract infection, typically by *E. coli*, <sup>148,149</sup> and

especially in women. It can also lead to bacteraemia. 150 A particular point of present interest regarding dormancy<sup>151</sup> is the fact that a high percentage of cystitis patients suffer reinfection, 152-159 that is often clearly from the same strains that caused the original infection. 160-164 This has led to the recognition in bladder epithelial cells of so-called 'quiescent intracellular reservoirs' 156, 158, 165-170 of dormant cells that can resuscitate. Because one cannot determine these things in humans in vivo, it is not known precisely how they enter such cells after binding to appropriate receptors such as uroplakins, 171 but it is presumed that as with many other cells where it is better understood this occurs via endocytosis of some kind.

Separating the blood from certain tissues are physical barriers such as the blood-brain, blood-retina and blood-testis barriers, consisting of layers of epithelial cells with especially tight junction. They are of notable significance to drug transport(ers) as well. 172-175 It is of particular interest that even here we can find that these barriers are (or must be) breached from time to time, as dormant microbes can be found even in the CNS. Possible means of resuscitation are discussed below and elsewhere.4

### A dormant CNS microbiome

As one might suppose, the CNS differs little from other tissues with regard to the possibility that dormant microbes may persist there, occasionally 'waking up' to cause trouble. Three examples, as they pertain to the aetiology of Alzheimer's disease (and presumably other dementias) are represented by Chlamydia pneumoniae (as stressed by Balin and colleagues, e.g. ref. 176-185), by herpes simplex virus (as highlighted by Itzhaki and colleagues<sup>30,88,182,186-195</sup>) and by a variety of spirochetes (as championed by Miklossy and colleagues 196-203). The latter is consistent with the well-established dementia in the terminal stages of another spirochetal disease in the form of syphilis, and also with Lyme disease. 199,204-206 Of course multiple classes of microorganisms may contribute. There is also evidence for a CNS involvement of the parasitic protozoan Toxoplasma gondii in a numbers of neurodegenerative diseases. 207-209

The 'gut-brain axis' describes the well-established observations of a bidirectional neurohumoral communication system in the human body. Given the above, it is not surprising that there has been increasing recognition over the last couple of years of quite overt (and bidirectional) communication between the gut microbiome and the CNS via the gut-brain axis, in particular, the idea that bacteria in the gastrointestinal (GI) tract can activate neural pathways and CNS signalling systems, from the earliest moments in life. Dysfunctions of this axis can lead to all kinds of neurological problems, including anxiety, depression and other CNS disorders (e.g. ref. 210-229). Most studies have focussed on the endocrine and immune systems. As yet, however, we have found no literature that has sought a role for LPS here, although given that LPS is actually used in a variety of experimental models (e.g. for Parkinson's<sup>4,230–237</sup> and even obesity<sup>238</sup>) to initiate CNS disorders, it is an easy

prediction that it is likely to play a significant role in the gut microbiota-brain interaction.

### A high-level overview of the effects of LPS in sepsis, septic shock and SIRS

Although our focus here is more on chronic inflammatory states induced by dormant and resuscitating bacteria, it is instructive first to consider events that occur in the more extreme and life-threatening cases of sepsis, septic shock, and the systemic inflammatory response syndrome (SIRS). While these are commonly observed in the Intensive Therapy Unit as a result of an initial infection (hence the term 'sepsis'), their typical treatment there with broad spectrum antibiotics means that proliferating microbes are rare or absent, and it is their products such as LPS that are then the main problem. Specifically, although these are responsible for invoking the innate immune response that triggers cells to attack and dispose of the invading microbes, an overstimulation of these activities leads to the life-threatening 'cytokine storms' that are the proximate causes of, and reflect, endotoxic or septic shock or SIRS (e.g. ref. 7 and 239-247).

It is worth rehearsing the definitions<sup>248,249</sup> to help discriminate sepsis<sup>250,251</sup> from its sequelae. Thus, sepsis has been defined as "the presence (probable or documented) of infection together with systemic manifestations of infection", 252 while severe sepsis is defined as sepsis plus sepsis-induced organ dysfunction or tissue hypoperfusion<sup>252</sup> Septic shock is "sepsis-induced hypotension persisting despite adequate fluid resuscitation". 252 SIRS refers to "the systemic inflammatory response to a variety of severe clinical insults" (infectious or otherwise). It usually involves two or more of the following criteria: (i) temperature > 38 °C or <36 °C; (ii) heart rate >90 beats per m; (iii) respiratory rate >20 breaths per m or PaCO<sub>2</sub> <32 mm Hg; (iv) WBC count > 12 000  $\mu L^{-1}$  or < 4000  $\mu L^{-1}$  or (v) > 10% immature neutrophil forms (i.e., "bands"). 253,254 The chief point about recognising and using SIRS instead of 'sepsis' is, of course, that it does not rely on the presence of observable (or culturable) microbes (and it can anyway be caused by traumas lacking an immediate microbial component). A Venn diagram (redrawn from ref. 254) illustrates the main ideas (Fig. 3).

Clearly any increases in microbial cell numbers increase the likelihood of LPS production and shedding that leads to the cytokine storm. Thus, the progression of the microbial variant in unfavourable cases goes roughly from left to right in Fig. 4, as infection  $\rightarrow$  bacteraemia  $\rightarrow$  LPS  $\rightarrow$  sepsis  $\rightarrow$  septic shock  $\rightarrow$ SIRS  $\rightarrow$  multiple organ failure (MOF/MODS)  $\rightarrow$  death. Mortality rates from sepsis/SIRS are extremely high (30-70% in intensive care units),241,242 and dependent on age.255 Note too that antibiotics can themselves promote shedding of LPS from dying bacteria (e.g. ref. 118 and 256-265), especially from spirochetes such as Borrelia burgdorferi, leading to a Jarisch-Herxheimer (JH) reaction.<sup>266,267</sup> The JH reaction can be mitigated by antibodies to TNF- $\alpha$ . 268 Importantly, this continual shedding of LPS is a normal property of growing Gram-negative bacteria, especially in

Integrative Biology Perspective

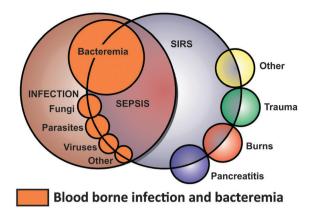


Fig. 3 Relationships and overlaps between bacteraemia, sepsis and systemic inflammatory response syndrome. Redrawn from ref. 254.

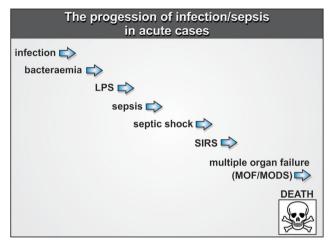


Fig. 4 The main steps that represent the progression of infection/sepsis in acute cases.

media such as those containing serum that are inimical to their growth. 257,269 This has obvious implications.

Even if one survives septic shock, there are other sequelae, such as long-term cognitive impairment, 270 cardiovascular 271 and other<sup>272</sup> complications that may reflect or contribute to symptoms seen under more chronic conditions.<sup>273</sup> Indeed, there are a great many comorbidities between various diseases with a microbial component.<sup>36</sup>

Clearly, LPS plays a central role in the development of inflammation. We therefore spend time in the next paragraphs to discuss the role of LPS in the cellular inflammatory processes.

### Positive feedback and amplification in integrative and systems biology

The first point to make here is a general one about how various kinds of kinetic schemes or network topologies can amplify biochemical signals. For a single enzyme, if its product or any other molecule is an uncompetitive inhibitor that binds only to the enzyme-substrate complex, a small amount of this can lead to a very large increase in a substrate concentration. This serves to explain both the peculiar effectiveness of glyphosate as a herbicide, 274-277 and the extreme rarity of uncompetitive inhibition in natural systems.<sup>278</sup> One type of network-based amplification, that is familiar in signalling cascades, is one in which a signalling activity such as a kinase changes the activity of another kinase, and so on. Here, as well as amplification, the cascade is partly about serving as a suitable delay loop, 279 as clearly variations in amino acid sequence can and do280 have major effects on the activities of individual proteins such that the cascade would otherwise seem unnecessary. A second kind of amplification, known as ultrasensitivity, comes from a structure in which an effector stimulates by covalent modification (e.g. phosphorylation) of an enzyme catalysing a particular reaction, while simultaneously inhibiting a second enzyme (e.g. a phosphatase) catalysing the removal of the covalent modification. This leads to very large changes in flux and network behaviour as the concentration of the effector passes a threshold.<sup>281-285</sup> Similarly, pulsatile or oscillatory signals can be much more effective for the same 'average' concentration. 286,287 Thus, a variety of network motifs can provide "sniffers, buzzers, toggles and blinkers". 288 And most of all, although other behaviours are possible, 289 a variety of simple systems with positive feedback can amplify a very small signal into a much larger one (Fig. 5). This can typically occur in inflammatory systems<sup>7</sup> where molecules whose production is induced by LPS, such as IL- $1\beta^{290-293}$  (Fig. 6) or TNF $\alpha$  (see later), can stimulate their own synthesis or effect crosstalk (e.g. IL-1 induced TNF- $\alpha^{294}$ ).

A second major area where an effector can appear to amplify a small stimulus, or have a large effect, is when it interacts with multiple targets simultaneously (in drug discovery this is known as polypharmacology (e.g. ref. 295-297)). This need actually follows from the principles of systems biology as encapsulated in metabolic control analysis, 298-301 where mediators that modify only one target can rarely be expected to have much effect. As we shall see, LPS qualifies here too, as it stimulates a great many proinflammatory and proapoptotic pathways, including those necessary for its translocation in both free and bacterial cell-associated forms.

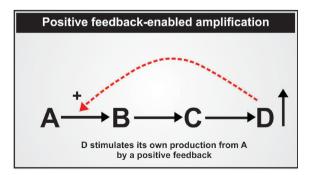


Fig. 5 A system exhibiting positive feedback in which a product stimulates its own synthesis. In a biochemical context, A to D represent metabolites, while the arrows represent enzymatic steps.

Inactive

Active
IL-Iβ precursor

IL-Iβ

Perspective Integrative Biology

Fig. 6 IL-1 $\beta$  is an example of a cytokine that can stimulate its own synthesis (figure redrawn from and based on one in ref. 291).

Thus we shall see that LPS is likely to serve as a major nexus in inflammation in general and that induced by microbes in particular.

### Structure and function of LPS

An overall 'cartoon'-type structure of typical LPS molecules is given in Fig. 7A based on. <sup>302</sup> They consist of an inner lipid A core and a number of branched polysaccharide chains (*e.g.* ref. 302–307), terminating in those that determine the strain's serology *via* the O-antigen. <sup>308</sup> The biosynthesis is discussed by Wang and Quinn. <sup>309</sup> According to the comprehensive LIPID-MAPS classification, <sup>310</sup> LPS is a saccharolipid glycan. The lipid A core is significantly the most inflammatory part of the molecule, <sup>311</sup> with typically two *N*-acetylglucosamine residues attached to a 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo) disaccharide (Fig. 7B). Bacteria lacking the outer O-antigen chains are known as 'rough' and are significantly more immunostimulatory than are their 'smooth' equivalents that contain them. <sup>312</sup>

## A systems perspective on LPS-induced cytokine production and cell death

A standard approach to systems biology modelling (*e.g.* ref. 279 and 289) has four main stages. The first two are qualitative, and involve determining the players and how they interact, whether as substrates, products or effectors – this establishes the topology of the network. The second two are more quantitative, involving the mathematical form of the equations describing each step, and their parametrisation, also involving the running of the model, typically as a set of coupled ordinary differential equations, using suitable software (*e.g.* ref. 313–315). Note too that in a typical network of this type, a systems approach typically discriminates parameters from variables. Parameters are either fixed or outside the control of the experimenter; they

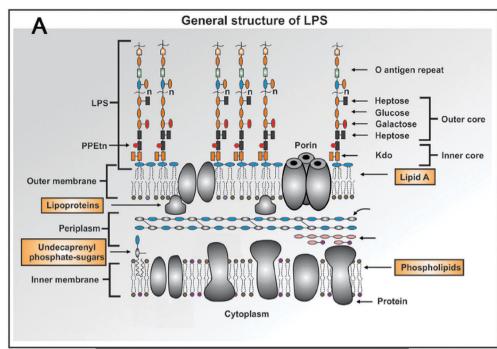
typically include the unchanging concentrations of substances involved in flux-generating steps, as well as kinetic constants such as  $K_{\rm m}$  and  $k_{\rm cat}$ . By contrast, variables are those things that vary during an experiment, typically involving concentrations of intermediary substances and fluxes through pathways towards 'exit' variables. Thus, figures such as those in Fig. 1, while accurate in the sense of illustrating flows of information, are misleading because they are at once both static and qualitative. While we can and shall point to many papers that show clearly that "LPS can cause inflammation (or apoptosis)", such a statement too is less than complete. This is because individual papers rarely if ever state, use or vary systematically a number of parameters that are known to have a huge impact on cell fate. These include

- The exact type of LPS (even though some, especially their lipid A component, are known to be much more immunogenic or inflammatory than are others see *e.g.* ref. 306, 309 and 316–324 and later)
- The rather variable amount of LPS added, whether the assay is for cytokine production (Table 3) or 'viability' (Table 4)
- The nature of the host (human or rodent, that respond differently, <sup>325–327</sup> or *in vitro*)
- Whether the LPS is added in a bolus or a dynamic manner (this matters a lot<sup>287</sup>)
- Whether measurements are done in single cells or as an ensemble, and or quasi-continuously; this matters because much evidence in the NF- $\kappa$ B system<sup>287,328-330</sup> and other related systems<sup>331-334</sup> shows that it is the nature and dynamics of the oscillations that determines which genes are transcribed and with which kinetics, as well as cell fate, and not just say an NF- $\kappa$ B concentration at a particular time. Thus knowledge of single-cell behaviour is vital<sup>3,110,111</sup> (also in pharmaceutical drug uptake<sup>174,175</sup>).
- Which other substances, conditions or parameters have been co-varied or even recorded, and whether they themselves are known to modify the effect of LPS alone. One example is ATP, which activates the purinergic PX27 receptors and increases massively the extent of cell death. Another is acidosis. Another is acidosis.

## LPS as a stimulant of inflammatory cytokine production: cellular mechanisms

While only partly consistent with the 'danger theory' of the immune response, <sup>364,365</sup> and more obviously stemming from the ideas of Janeway, <sup>366</sup> LPS is recognised as a major 'pathogen-associated molecular pattern' or PAMP that triggers the body's innate immune response to pathogens (*e.g.* ref. 367). In addition, cells release damage-associated molecular pattern molecules (DAMPs) as signals that alert the innate immune system to unexpected cell death and to microbial invasion. It is now very well established that a chief means by which LPS excites an inflammatory innate immune response is by binding to the toll-like receptor 4 (TLR4). <sup>368–373</sup> Typically, the LPS is bound in

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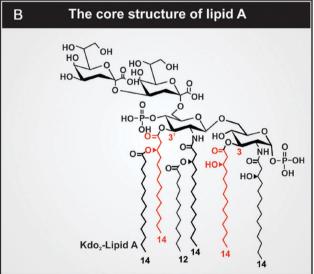


Fig. 7 The general structure of (A) LPS and (B) its core lipid A component. Redrawn from ref. 302.

blood to LBP, and the LPS is ferried to the TLR4 receptor *via* a CD14 co-receptor (which also links innate immunity with Alzheimer's disease<sup>374–377</sup>). This binding of LPS to TLR4 in turn activates the production of a variety of pro-inflammatory cytokines.<sup>378–381</sup> The extent of cytokine activation reflects in part the strength of binding to CD14/TLR4.<sup>320</sup> These inflammatory cytokines are induced *via* a set of canonical pathways illustrated in Fig. 8, with the transcription factor NF-κB playing a prominent role.<sup>382–385</sup> As is also well known (and see below), NF-κB is normally held inactive in the cytoplasm by being bound to an inhibitor IκB protein, and the means by which extracellular signals such as LPS are transduced involve a series of kinases, one of which (IKK) in particular phosphorylates the IκB and thereby releases the NF-κB that can translocate to the

nucleus to turn on a large variety of other genes, including in particular TNF- $\alpha$  and IL-6. There is also a 'non-canonical' inflammasome LPS activation pathway independent of TLR4, 387–389 that occurs at higher external concentrations of LPS, 23,390 comes into play when the LPS is internalised, and involves (*via* p38 MAP kinase and intracellular LPS) the activation and secretion of cytokines such as IL-1 $\beta$  (Fig. 9) and also TNF- $\alpha$ ).

### Muropeptides and host signalling

A more recent recognition is that as well as lipid A, shorter bacterial cell-wall derived muropeptides also have a significant role in innate immunity (*e.g.* ref. 392–405; they act synergistically

Table 3 Some examples of LPS administered to cells (primary or permanent cell culture or in vivo) or animals and its effect on interleukin and TNF-a production

Cell type/animal origin	Cell name	LPS concn administered to cells/animals	LPS type	Measurement: interleukin and TNF- $\alpha$ production	Ref.
Mouse origin Peritoneal mouse macrophages C57BL/6 mice	Raw 264.7 mouse macrophages Primary microglial cultures	$100~{\rm ng~mL^{-1}}\\100~{\rm ng~mL^{-1}}$	Escherichia coli O55:B5 E. coli O26:B6	Increased IL-6 production Massive increase in IL-1β when purinergic	338 335
IL-1Ra knockout (KO) and wild-type (WT) mice	Peritoneal macrophages (primary cultures)	$10~\mu{ m g~mL}^{-1}$	Aggregatibacter actinomycetemcomitans	receptor activated In-reased production of IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ and IL-6 in IL-1Ba KO mice, compared with	339
Mouse alveolar macrophages	MH-S cell lines	$100~{\rm ng~mL^{-1}}$	Sigma LPS type	Lie levels in W 1 mice. So stimulated overproduction of TNF- $\alpha$ and	340
C57BL/6 mouse Mouse microglia	Primary macrophages BV2 microglia	$1~{\rm \mu g~mL^{-1}} \\ 100~{\rm ng~mL^{-1}}$	not stated E. coli O26:B6 E. coli O55:B5	IL-10 Massive increase in IL-1 $\beta$ but not IL-6 LPS-stimulation induces the expression of	341 342
Intact mice $(ob/ob)$ , or C57BL/6		$5~\mathrm{\mu g}~\mathrm{or}~100~\mathrm{\mu g}~\mathrm{kg}^{-1}$	E. coli O127:B8	Massive increases in $IL-1\beta$ , $IL-6$ and $IL-RA$	238
with diet-induced obesity J774.A1 macrophages	Primary cultures	$10~\mu \mathrm{g~mL}^{-1}$	Not stated, but was $E.\ coli$ O26:B6	Huge increase in IL-1 $\beta$ production	343
Rat origin Rats Sprague-Dawley rats hippocampal brain slices	Serum level measurement Primary culture	100 µg per rat, iр 10 µg m $ m L^{-1}$	Not stated E. coli O55:B5	Increased IL-6 and TNF- $lpha$ production Significant increased concentration of TNF- $lpha$ and IL-1 $eta$	344 345
Human origin Human bone marrow aspirates	Bone marrow mesenchymal	$0$ to 10 ${ m \mu g~mL^{-1}}$	Porphyromonas	IL-6 production independent of the LPS	346
Peripheral blood mononuclear cells	Stem cell primary cultures Primary culture white	0.1, 1 or 100 ng m $\mathrm{L}^{-1}$	gingwans LPS Not stated	dosage II6 and TNF-α were both strongly	347
HepG2 Peripheral blood mononuclear cells (PBMC) (from healthy and	Diood cells Hepatoma cell line Primary culture white blood cells	$0.1$ , 1 or 100 ng mL $^{-1}$ 2 and 0.2 ng mL $^{-1}$	Not stated TLR4 ligands LPS	upregulated Slight inhibition of TNF- $\alpha$ Slight inhibition of TNF- $\alpha$ With low LPS dose the T2D cohort exhibited enhanced IL-1 $\beta$ relative to healthy cells	347
type 2 diabetes individuals (12D)) Nasal polypfibroblasts	Primary cultures	$10~\mu \mathrm{g~mL}^{-1}$	E. coli O111:B4	LPS enhanced the secretion of IL-6	349
Other animal origin Airway neutrophilia was induced in horses by inhalation of LPS	Animal model	$1~{ m mg~mL^{-1}}$ per horse	Sigma LPS type not stated	Significant increased concentration of IL-6 and TNF- $lpha$	350

Table 4 Some examples of LPS administered to cells (primary or permanent cell culture or in vivo) and its effect on cell viability

Cell type	Cell name	LPS conc (ng $mL^{-1}$ )	LPS type	Viability assay	Viability %	Ref.
Rat origin						
Rat duodenum	Epithelial cells	0.75-3 mg kg <sup>-1</sup>	H. pylori LPS	MTT	60%	351
Rat myocytes		i.v. or 3–12 mg kg <sup>-1</sup> p.o 25–10 000 (most 100)	Unstated	Apoptosis	80%	352
Alveolar macrophages	NR8383	10	Unstated	LDH and Hoechst/PI	90% without particulates	353
Myocardial myocytes	H9c2	1000		MTT, LDH, TUNEL, JC-1	85%	354
Myocardial myocytes	H9c2	20 000		MTT	65%	355
Mouse origin						
C57BL/6 mice	Primary microglial cultures	100	E. coli O26:B6	LDH release	~10% in presence of ATP	335
Osteoblast	MC3T3-E1	10 100 1000	E. coli O55:B5	MTT	70%	356
Mouse macrophages	RAW 264.7	100	Unstated	MTT	Not stated; increased apoptosis	357
Macrophages	BMDM	100 (6 h)	Unstated	DNA fragmentation	~60%	358
Human origin						
Pulmonary epithelia	A549	1000	Unstated Sigma	Unstated kit	60-70%	359
Human PBMC	PBMC	0.01-3	E. coli unstated	None - only IL-8 and ROS		360
Microgial	BV2	10 000-30 000	Unstated	MTT and trypan blue	~60%	361
Vascular human endothelia	HUVECs	500	Unstated	Annexin	80%	362
Dopaminergic	SH-SY5Y	100	E. coli O127:B8	MTT	Unclear	363

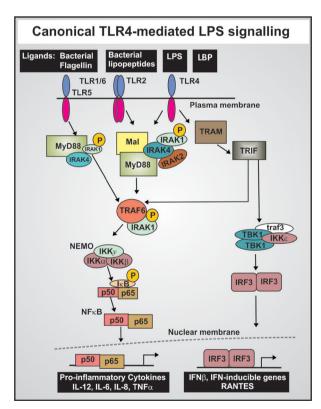


Fig. 8 The LPS-mediated cellular production of inflammatory cytokines. Canonical pathway of LPS-mediated release and nuclear translocation of NF-κB (based on ref. 379).

with LPS, 396 probably because they also interact with the NF-κB pathway, via the RICK/Rip2/CARDIAK kinase406,407). It is of particular interest in the context of bacterial dormancy that such muropeptides seem to be part of the 'wake-up' activity of the

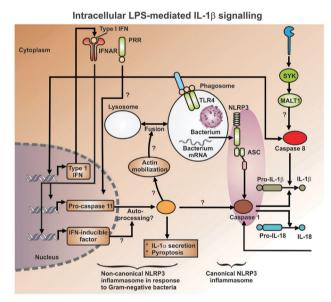


Fig. 9 The intracellular LPS-mediated activation of caspase-1 leading to IL-1β production (after ref. 391).

bacterial Rpf408 and other bacterial resuscitation systems.409 See Fig. 10 for a visual representation of the host signalling pathway of MDPs.

### LPS induction of apoptotic, programmed necrotic and pyroptotic cell death

A particularly important inflammatory cytokine whose secretion is induced by NF-κB (and also by p38 MAP kinase) is

CD14

MDP

SLC15

NOD2

TRAF6

TAB2

TAK1

TAK1

IKKY

IKKΩ

IKKΩ

IKKΩ

IKKΩ

IKKΩ

IKKΩ

Cytoplasm

Fig. 10 Host signalling pathways of muramyl peptides (MDP). Based on and redrawn from ref. 405. MDP are taken up *via* the pepT1 oligopeptide transport system (SLC15 – see ref. 410).

TNF- $\alpha$  (e.g. ref. 411-414). This too is inflammatory and itself induces further changes in NF-kB expression. The same is true for IL-1<sub>B</sub>. 343,378 In particular, these inflammatory cytokines can lead to apoptotic cell death (e.g. ref. 343 and 358). As we shall see, however, and as presaged in Tables 3 and 4, it is quite difficult to establish precisely what is going on in many cases, as individual studies tend to study a restricted set of pathways and individual players, be they NF-κB, p38, IL-1β or IL-6. It would not in our view be completely unfair to describe a lot of these studies collectively as 'a bit of a mess'. Taking NF-kB as a canonical example (though the same is true for p38<sup>415</sup>), what are ostensibly the same kinds of signal can lead to dramatically different cell fates, e.g. proliferation vs. apoptosis416-424 depending on the conditions. What this is telling us, of course, since these processes are considered largely deterministic, is that we are not measuring or controlling all of the relevant factors (see also Table 3), and in great measure these studies are largely qualitative in nature. Here our purposes are thus simply to recognise that the genes induced or repressed via transcription factors such as NF-κB can be pro- or antiapoptotic, and which ones are activated depend on all prevailing

As well as apoptosis, there is a less tightly (but partly) regulated form of cell death known as 'programmed necrosis' or 'necroptosis', 425-437 that may be induced by inflammatory

ligands such as TNF, especially during infection, and that sometimes also involve NF- $\kappa$ B. Another important mode of cell death induced by related stimuli is pyroptosis<sup>438–450</sup> (that involves the caspase 1-dependent production of IL-1 $\beta$ ). Ferroptosis<sup>451,452</sup> is a cell death mechanism that stresses the importance of unliganded iron (see also ref. 6, 7, 453 and 454). Although the stimulus in each of these cases is nominally the same (LPS of some kind) there are presumably pre-existing conditions that differ and thereby determine precisely the kind of cell death that ensues. However, we do not discuss the emerging differences in their molecular details nor taxonomy here, since the important thing for the present arguments is simply that the cells die, disappear, and thereby leave gaps where once they lived.

## LPS as a stimulant of coagulation and thrombosis

As we recently reviewed, <sup>9</sup> a hallmark of many chronic, inflammatory diseases is the fact that they simultaneously exhibit both hypercoagulability and hypofibrinolysis. While a great many biochemicals can influence both the kinetics and endproduct structures of the clotting process, and we previously highlighted unliganded iron<sup>8,10,455-458</sup> and the fibrin concentration itself, <sup>9</sup> we can hardly avoid noting that LPS itself is a strong procoagulant.<sup>459-463</sup> How direct some of these mechanisms are seems not to have been established, though certainly LPS can bind to erythrocyte membranes.<sup>464,465</sup> Given that promiscuity correlates with hydrophobicity (*e.g.* ref. 173 and 466–469), it is not surprising that the very hydrophobic LPS can potentially interact with a great many (lipo)proteins; its ability to convert prions to their more toxic PrP<sup>Sc</sup> form<sup>470</sup> is a pertinent case in point.

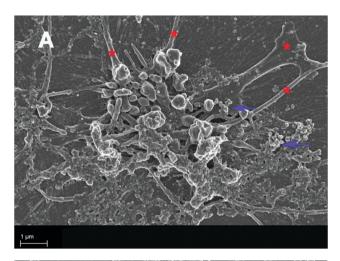
### The role of microparticles and LPS

We wish, however, to spend some time on the possible involvement of LPS in microparticle formation. Microparticle formation is typically via apoptosis and the related pathways described above. 471-474 Such microparticle formation is prominently associated with inflammatory conditions. Microparticles have also been associated with proinflammatory effects and also with autoimmune processes, and they are thought to be a source of autoantigenic nuclear material, which can form immune complexes. 475 Various cells such as platelets, lymphocytes, endothelial cells, erythrocytes and monocytes do release surfacederived microparticles476 and these microparticles are seen as multi-purpose carriers. 477 They carry proteins, lipids and nucleic acids, and play a fundamental role in the pathogenesis of thrombosis and are known to modulate the properties of target cells.478 Microparticles from erythrocytes also carry heme and these heme-laden microparticles have a physiopathological impact on the rest of the haematological system. 479 Microparticles also frequently elicit an immune response.  $^{480\mbox{-}483}$ 

As microparticles are known to be present in many inflammatory diseases,  $^{8,478,484-487}$  they might therefore develop via an

Integrative Biology Perspective

external or internal stimulus on cells (e.g. erythrocytes and platelets), and here we suggest that the stimulus might be LPS. While pure lipid systems are not always good membrane mimics, 172-174 LPS has been shown to insert spontaneously into lipid bilayers, and this insertion can lead to membrane breakdown. 480,488,489 This insertion capability has also been demonstrated in lipid raft models. 490 Given that we have recently shown that bacteria can hide inside erythrocytes, shedding of LPS may thus occur within the cells (as well as obviously outside the cells, where "free" bacteria may shed LPS). There is considerable literature that suggests that LPS can be a cause of apoptosis etc, so LPS shed from internalized



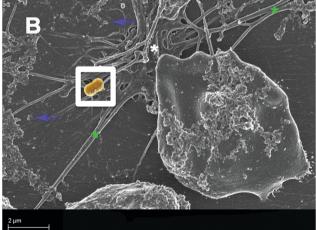


Fig. 11 Whole blood smears from a thrombo-embolic ischemic stroke patient. (A) Hyperactivated platelet mass with pseudopodia and microparticle formation. (B) Hyperactivated platelet mass showing fusion with spontanously formed fibrin fibres, in whole blood. This activated mass is closely associated with an abnormally shaped erythrocyte covered with plasma proteins/microparicles, either from apoptotic hyperactivated platelets or damaged erythrocytes. Explanation of asterisks and arrows: in (A): red asterisk: platelet pseudopodia formation; blue arrows: microparticles. In (B): blue arrows: microparticles; green asterisk spontaneous fibrin fibre formation merging with pseudopodia formation from a hyperactivated, spreaded platelet (white asterisk). Note significant pseudopodia/ fibrin extending from the spreaded platelet mass (white asterisk). Bacterium (pseudo-coloured in yellow-brown) is shown with a white block drawn

cellular bacteria may also trigger apoptotic pathways from within the cells (via caspase-11, caspase-4 and IL-1β pathways), resulting in microparticle formation. This eventually stimulates the processes of coagulation and thrombosis already known to be associated with microparticle presence in inflammatory conditions. 491,492 An example of this is shown in Fig. 11A and B, where microparticle formation in thrombo-embolic ischemic stroke is seen associated with both hyperactivated platelets and damaged erythrocytes, together with the presence of bacteria.

### The sequelae of intestinal inflammation and LPS-induced apoptosis, necroptosis and pyroptosis include epithelial permeabilisation

It is clear that as the concentration(s) or activities of LPS, inflammatory cytokines and other mediating factors increase, cell death is an inevitable consequence. 425,493-495 While, as mentioned above, there is almost certainly a continuing small leakage of microbes from the gut496 (known as the 'leaky gut' hypothesis), it is evident that a variety of conditions, that we may loosely refer to as 'stress' can increase this considerably (e.g. ref. 73, 78 and 497-505). Bacterial sepsis itself is one such stress, 506 and interestingly there is now a burgeoning literature to the effect that the immunodeficiency seen in HIV/AIDS patients may actually be caused by gut-derived LPS causing hyperactivation (then death) of CD4<sup>+</sup> cells (e.g. ref. 138 and 507-509). There does not seem to be a major genetic contribution to leaky gut. 510 Although many of the same signalling pathways are involved, the extent to which this is mediated via LPS is not yet clear, albeit LPS itself can indeed disrupt tight junctions and increase intestinal permeability.511-516 Overall, these kinds of endothelial dysfunction clearly lead to increased leakiness or permeabilisation 430 (Fig. 12).

### Localised microbial proliferation, inflammation and cell death as a cause of specific diseases

We have here sought to provide a rather general explanation for the role of LPS-induced chronic inflammation in a variety of different diseases, with the 'continuing' element driven by the resuscitation of a resident blood and tissue microbiome. However, it is obvious that while all these diseases are inflammatory (and have been linked to microbes<sup>4,5</sup> and/or LPS (Fig. 2), diseases such as Alzheimer's, Parkinson's, atherosclerosis and rheumatoid arthritis obviously occur - or one might better say manifest in largely different tissue or tissue subtypes. Thus, the disappearance of cells from the CNS can manifest as Parkinson's disease if in the dopaminergic neurons of the substantia nigra pars compacta,517 or to more widespread cell disappearance is diseases such as those mediated by prions (e.g. ref. 7 and 518). Note of course that LPS is actually used to induce a form of Parkinson's disease in experimental animals. 4,230-237

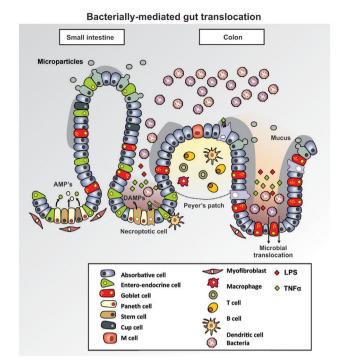


Fig. 12 Some of the mechanisms of bacterial translocation from the intestine in a 'leaky gut', based on ref. 430. Some cells (and LPS) may pass through tight junctions, while others pass through Peyer's patches or through spaces vacated by dead cells whose death may be initiated by LPS. Diagram is not to scale.

Within the present framework, there are at least three general and straightforward explanations for this differentiation of diseases despite a broad common cause. The first is that (as with infectious diseases), the nature of the microorganisms differs, with some of these inflammatory diseases clearly being more associated with some microbes than are others (e.g. ref. 5, 43 and 64). The second is simply that the tissue location of the dormant bacteria differs. The third follows as much from our ignorance of the molecular details, as discussed above, as from what we know - namely the fact that we know little of the different effects of LPS type and concentration, and the effects of different levels of cytokines and other molecules that may themselves vary this. Sharpening these kinds of analyses will obviously feature as an important area for future research, and we rehearse the first two points briefly.

### Differences in the organisms forming the dormant blood microbiome

We<sup>5</sup> and others (e.g. ref. 36, 43, 48, 49, 52, 62, 64 and 519–522) have provided both (ultra)microsocopic and molecular (sequencebased) evidence for a very great variety of culture-negative organisms that have been noted to be present in the blood, despite the fact that it is normally considered to be sterile.

### Differences in the tissue locations of dormant bacteria

In a similar vein, microbes - presumably persistent, dormant organisms - are regularly detected in other tissues in which any

degree of proliferation would be highly inimical to the host. Some recent examples involving just reproductive disorders include the vagina, 523-525 the placenta, 526-530 and the amniotic fluid, 531-540 while recent evidence has also been provided for a sub-epidermal microbiome. 541 The considerable evidence for a dormant CNS microbiome was discussed earlier, and we here note the presence in the brain of TLR4 and its major role in neurodegeneration, 542 consistent with the idea that waking up dormant microbes can stimulate overt neurodegenerative disease.

### A note on autoantibodies

A number of the diseases (e.g. rheumatoid arthritis, multiple sclerosis, psoriasis) for which we are invoking a microbial component involving LPS are usually considered to be autoimmune diseases. The question then arises as to the origins of this autoimmunity. If LPS were a protein with a defined structure it would be relatively easy to compare its epitope sequences with those of the targets of host antibodies in different circumstances (e.g. ref. 543-546), but of course it is not. This said, there is plenty of evidence that host autoantibodies are elicited by LPS that have less than perfect specificity for the immunogen (e.g. ref. 547-558), so while most of this work is not very recent, this question of LPS-induced antibody non-specificity seems an avenue well worth exploring. We note too the potential toxicity of exogenously administered anti-LPS antibodies. 559,560 Regarding an autoimmune hypothesis, Marshall, Proal and colleagues highlight precisely this, along with a role for the vitamin D receptor. 35,36,561-565

### Other hallmarks, and the role of iron dysregulation

While LPS itself as commonly measured seems to be a rather inadequate biomarker for chronic, inflammatory disease (Table 2), LBP (Table 1) and longer-lived markers of LPS exposure like IgM and IgA antibodies<sup>4,543–545,566–570</sup> may be more promising.

What serves to wake up the dormant microbes is not yet clear, and it is unlikely to be a single element. One possibility is certainly host stress as reflected in noradrenaline levels, a mechanism championed with considerable evidence (at least in terms of stimulating the growth of Gram-negative organisms) by Lyte, Freestone and colleagues. 215,222,571-579 We note that catecholamine synthesis may be induced in macrophages by LPS, 580,581 and that catecholamines increase inflammation 582,583 (another positive feedback loop). It is of special interest here that noradrenaline can act as an iron chelator, 584-586 since iron is normally seen as the nutrient most limiting to bacterial growth in vivo (e.g. ref. 16, 19-21 and 587-591). As we have pointed out before,6-10 the diseases highlighted here are precisely those where iron dysregulation, inflammation, coagulopathies, microparticles, 592,593 and now a microbial component involving LPS sensu lato, are seen to coexist.

Concluding remarks, areas of

Integrative Biology

## ignorance, and therapeutic potential

It is undoubtedly the case that despite our greatly improved understanding of many of the pathways involved in inflammation and sepsis (sensu lato), strategies for drug development have been noted mainly by their relative failure, 411,594-597 probably in significant measure because they aim at individual targets (see ref. 297). As a systems problem, the way to improve our therapeutic strategies is to recognise that there are multiple targets or steps. We have here stressed that LPS (sensu lato) created by the resuscitation of dormant bacteria is likely heavily involved, and that the latter can be affected greatly by the concentrations of free iron (that may also be a target for therapy). Fig. 13 shows some of the various translocations of dormant and proliferating bacteria between the different tissues, and the various steps that might thus be targeted. Improving methods for quantitative lipidomics (e.g. ref. 598–603) are likely to be of considerable value here.

Here we have stressed the significance of LPS, but initially promising trials of an anti-LPS antibody (HA-1A, Centoxin)<sup>604</sup> were not sustained (as is common<sup>605,606</sup>) because of ineffectiveness<sup>607,608</sup> or toxicity. <sup>559,560</sup> This was probably because of its lack of specificity between lipid A and other hydrophobic ligands, 609 and drug promiscuity is a function of hydrophobicity more generally (e.g. ref. 173, 468 and 610-612). Recognising that factor C, the active component of the Limulus amoebocyte lysate, 94 necessarily binds to LPS, Ding, Wohland and colleagues have isolated so-called sushi peptides therefrom, 613-620 in particular

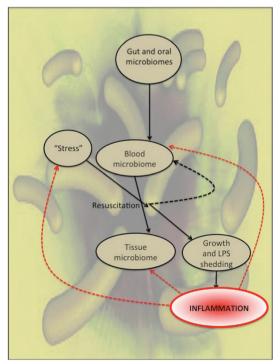


Fig. 13 Some of the various translocations of dormant and proliferating bacteria between different tissues as discussed herein. The red dotted lines indicate the influence of inflammation, while the resuscitation step is indicated by the black dotted line. The solid black lines likely involve some kind of translocation

sushi peptides S1 and S3. These are 34mers that that can bind to and inactivate endotoxin molecules (and are in some cases directly antibacterial), in concert with bacterial phospholipids. 621 They are said to have low human cell toxicity, and these peptides (or variants<sup>622,623</sup>) would seem to have significant therapeutic potential. As a hydrophobic target, variant lipocalins binding LPS ('anticalins' 624-628) might also be of value here. The evidence that HDL can bind to and sequester LPS was given above.

It is hard to gauge whether a better treatment than an anti-LPS antibody might involve recombinant LBP because while LBP can sequester LPS it can also transfer it to TLR4. 127,134,629-631 Probably the best strategy is to avoid letting the bacteria proliferate in an uncontrolled manner at all, and to this end an ironwithholding strategy<sup>632</sup> seems most suitable (e.g. ref. 6, 7 and 633-636) (and not a fortification one<sup>637,638</sup>). In a similar vein, many of the substances found to be anti-inflammatory are so because of their iron-chelating properties (e.g. ref. 7 and 639-641), though clearly there are a vast number of possible steps (plural) at which anti-inflammatory substances may act. Those involving downstream cytokines (e.g. ref. 539) are outwith our scope here.

In addition, provided one can avoid excessive LPS shedding and a Jarisch-Herxheimer reaction, 266,268 suitable antibiotics (e.g. minocycline<sup>642-650</sup>) that have polypharmacological properties<sup>651,652</sup> must certainly have a role in treating purportedly non-communicable inflammatory diseases, as well as those established as infectious. It is at least worthy of mention, 653 albeit drugs can be quite promiscuous with regard to hitting multiple targets, 173,466,651,652,654 that certain antibiotics are both used and effective in the treatment of diseases such as multiple sclerosis, 655-661 rheumatoid arthritis, 544,642,651,652,662-665 and psoriasis, 666-668 while vaccination can also work to protect against supposedly non-communicable disease. 669,670

A modern trend is to use patients as their own controls (so-called  $n = 1^{671}$  or n-of- $1^{672,673}$  methods), in suitable crossover designs. These seem ideally suited to chronic diseases of the type discussed here, especially for those in which disease severity can itself change significantly ('flare', 674-677') on quite rapid timescales (for the large and very numerous circadian changes in a mammalian transcriptome, see ref. 678). Indeed, we predict that such flares will be accompanied by major changes in the relevant microbiomes. But to understand the precise mechanisms involved we shall need much better, more quantitative, and above all reliable methods for measuring and manipulating the microbes, pathways and pathway elements involved. This is the most urgent task for the future.

### Note added in proof

A recent and complementary article highlights the role of LPS translocation directly from the gut.679

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