TIMELINE

Innate immune sensing and its roots: the story of endotoxin

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How does the host sense pathogens? Our present concepts grew directly from longstanding efforts to understand infectious disease: how microbes harm the host, what molecules are sensed and, ultimately, the nature of the receptors that the host uses. The discovery of the host sensors — the Toll-like receptors — was rooted in chemical, biological and genetic analyses that centred on a bacterial poison, termed endotoxin.

In all epochs and in all cultures, humankind has been tormented by the sudden and widespread appearance of lethal illnesses, which we know today as infectious diseases. The multifarious nature, unpredictability and deadly power of plague, cholera and typhoid made these diseases a constant threat to society. Medicine and science were much challenged to identify the means by which such diseases originated, spread and killed human beings, without regard to their social status, ethnic origin, religion, sex or age.

A central question concerned the ultimate cause of death. Unless diseases were seen as an expression of the will of God, only the identification of their lethal essence would enable the development of preventive or therapeutic measures. Because some disease symptoms, such as fever, vomiting and diarrhoea, were also seen after intoxication, the schools of Hippocrates (ca. 460–370 BC) and Galenos (Galen of Pergamum; ca. AD 129–199) concluded that a poison was the proximal cause of illness (FIG. 1). But what was this poison? The competing theories of 'miasma' and 'contagion' formed the conceptual framework within which early workers sought to identify it.

Impressed by the malodorous exhalations of patients suffering from plague and their similarity to the foul vapours emanating from marshes, physicians came to believe that the putative poison was generated by putrefaction (from the Greek for sepsis) of organic matter present in sick people or in locations such as swamps. The bad air of the marshes (nowadays still present in the term malaria = mal'aria) was named miasma (from the Greek 'miainein' meaning 'pollute') and was believed to spread the poison, thereby plausibly explaining the death by inhalation of thousands of people within a short period of time. The miasma model could not, however, explain why the isolation of sick people for 40 days (quarantine) often prevented the spread of the disease — surely, all people must breathe the same air, whether or not the sick were isolated from those who were well.

An alternative belief, therefore, was that a non-volatile poisonous material caused the disease. This material, termed contagion (from the Latin 'contigere' meaning 'to touch'), was also thought to be produced by putrefaction of organic matter such as meat, but to be transmitted only by direct contact.

The hypothesis that a veritable poison was present in putrid matter received strong support from the experiments of Albrecht von Haller (1708–1777) and François Magendie (1783–1855), who showed that intravenous application of decomposed fish or meat to experimental animals caused symptoms of illness[1,2]. Extracts of organic matter that had not undergone decomposition failed to have such effects. In attempts to isolate and characterize the poisonous material, Peter L. Panum (1820–1885) could be considered a pioneer. He showed that putrid fluids contained a water-soluble, but alcohol-insoluble, heat-resistant, non-volatile substance, which was lethal to dogs[3]. Also, Ernst von Bergmann (1836–1906) believed that a chemically defined substance was responsible for putrid intoxication, which he termed sepsin[4].

Of course, the contagionists could not explain how a single contact with putrid fluids or a sick patient could transmit so much poison that not only the affected person, but also thousands of other people, would die. It was, therefore, an intellectual breakthrough to postulate that the putrid venom communicated by miasma or contagion could reproduce in the affected individual, thereby having attributes of a living organism. This revolutionary idea was formulated by Jacob Henle (1809–1885), who without knowing about microbes, stated: "One atom of smallpox venom is capable of causing a rash of variola over the whole body," thereby indicating multiplication of the toxic matter[5]. So, Henle, who was later to be a teacher of Robert Koch (1843–1910), promoted 'microbiology without microbes' and stood at a point of transition between the pre-microbial and the microbial eras.

Chemical resolution of the poison(s)

It was Louis Pasteur (1822–1895) who proved beyond any doubt that it was germs — and nothing else — that were responsible for the putrefaction and decomposition of organic matter. He recognized that microbes (the term 'microbiology' was created by Pasteur) were not only necessary, but also sufficient, to cause an infectious disease[6]. Robert Koch then showed that a given infectious disease, such as tuberculosis or cholera, was caused by a specific living microorganism, which, after entering the body, multiplied and caused the disease, together with all of its symptoms[7]. So, the 'poisonous' principle was embodied in

microbes that were present in putrid matter (contagion) or in droplets expectorated by infected patients (miasma). However, the mechanisms by which the microbes exerted their damaging actions in the host remained unexplained. In particular, infections often resulted in toxicity that was out of all proportion to their apparent severity: a small amount of infected tissue could have serious consequences for the individual.

The discovery that germs produce and secrete poisonous products, termed 'toxins', in 1886 by Ludwig Brieger (1849–1919) was a further intellectual advancement[8]. Diphtheria toxin and tetanus toxin were among the first bacterial poisons to be identified. They were found to be present in culture supernatants, to be sensitive to heating and to be subject to neutralization by substances produced in experimental animals that were injected with the toxin. Such findings culminated in the discovery of anti-toxins by Emil von Behring (1854–1917) and Shibasaburo Kitasato (1856–1931) in 1890. The term 'antibody' was created in 1891 by Paul Ehrlich (1854–1915). However, in supernatants of living cultures of *Vibrio cholerae*, the causative agent of cholera, a heat-labile toxin could not be identified. Robert Koch, who had identified *V. cholerae* in 1884, nevertheless postulated that a poisonous substance had an essential role in cholera pathogenesis[9,10]. Given these circumstances, Koch seems to have encouraged one of his co-workers, Richard Pfeiffer (1858–1945), to examine the nature of the toxins involved in cholera pathogenesis. During his studies, which involved the extra-enteric inoculation of *V. cholerae*, Pfeiffer discovered a phenomenon that made him world famous, and that carries his name[11]. After inoculating guinea pigs that had been either actively or passively immunized against *V. cholerae*, and waiting, he found that no living *V. cholerae* organisms could be detected in the abdominal cavity. Yet, remarkably, the animals would die. Apparently, the bacteria had undergone lysis, indicating that *V. cholerae* toxicity was not dependent on bacterial viability, but resulted from the action of a bacterial poison that was normally contained in the bacterial cell but that was released during bacteriolysis.

Pfeiffer then showed that cholera bacteria that had been killed by heat retained their toxic potential, which proved that the poison was not a classical protein toxin. His experiments led him to formulate the concept that *V. cholerae* harboured a heat-stable toxic substance that was associated with the insoluble part of the bacterial cell[12,13]. He called this substance endotoxin (from the Greek 'endo' meaning 'within'). Pfeiffer proposed that endotoxins were constituents of nearly all groups of bacteria — both Gram-negative and Gram-positive — and he identified them subsequently in *Salmonella typhi* and *Haemophilus influenzae* (a bacterial genus that Pfeiffer himself had discovered).

In time, endotoxin became the focus of a vast inquiry into the molecular mechanisms of microbial pathogenesis[2,14,15]. The Italian pathologist Eugenio Centanni (1863–1948), who was conversant with microbes and with Pfeiffer's work, summarized Pfeiffer's work by stating: "Thus, we can conclude that the whole family of bacteria possess essentially the same toxin … upon which depends the typical picture of the general disturbances caused by bacterial infections"[16]. Also, Centanni recognized the intimate relationship between the pyrogenic and toxic properties of the poison, which he found to be chemically inseparable. This caused him to name his material 'pyrotoxina' (from the Greek 'pyros' meaning 'fire').

Fever had been known for decades as a symptom of disease, or even as a disease in itself, but it was starting to be recognized as beneficial to the host. William B. Coley (1862–1936) showed that mixtures of killed bacteria (*Serratia marcescens* and *Streptococci*) not only caused fever, but also induced remissions of certain malignant tumours in humans[17]. This formed the background for the discovery of tumour-necrosis factor (TNF) many years later. In addition, 'fever therapy' was effective for treating psychiatric conditions and certain infections (notably syphilis), and for stimulating the immune system in general. So, the realization that putrid poisons were not only detrimental, but also beneficial, to the host began to dawn nearly a century ago. This tentative connection between microbial toxins and the system for innate immune recognition would grow to form a solid conceptual link in the 1970s, buttressed by strong experimental data, as described below.

Figure 1 | **Hippocrates and Galenos, the leading medical doctors of their times and creators of the theory that many diseases are of poisonous origin.** Thirteenth century fresco in the crypt of the Domo of Anagni near Rome, Italy. The theoretical and therapeutic concepts of these two masters dominated European medical education and practice until the seventeenth century. The text on the paper in front of Galenos (left) reads "Mundi presentis seres manet ex elementis", meaning "The present world's connection persists on the basis of the elements". The text on the paper in front of Hippocrates (right) reads "Ex his formantur que sunt quecu(m)q(ue) chreantur", meaning "Of these (all) is formed which exists and which will be created". The four basic elements were represented by air, fire, earth and water. Image courtesy of the Art Archive/Anagni Cathedral Italy/Dagli Orti (A).

Chemical definition of endotoxin

Largely through the work of Mary Jane Osborn and Hiroshi Nikaido, we know today that endotoxin is an important structural component of the outer leaflet of the outer membrane of Gram-negative bacteria[15], containing the O-antigenic polysaccharide determinants

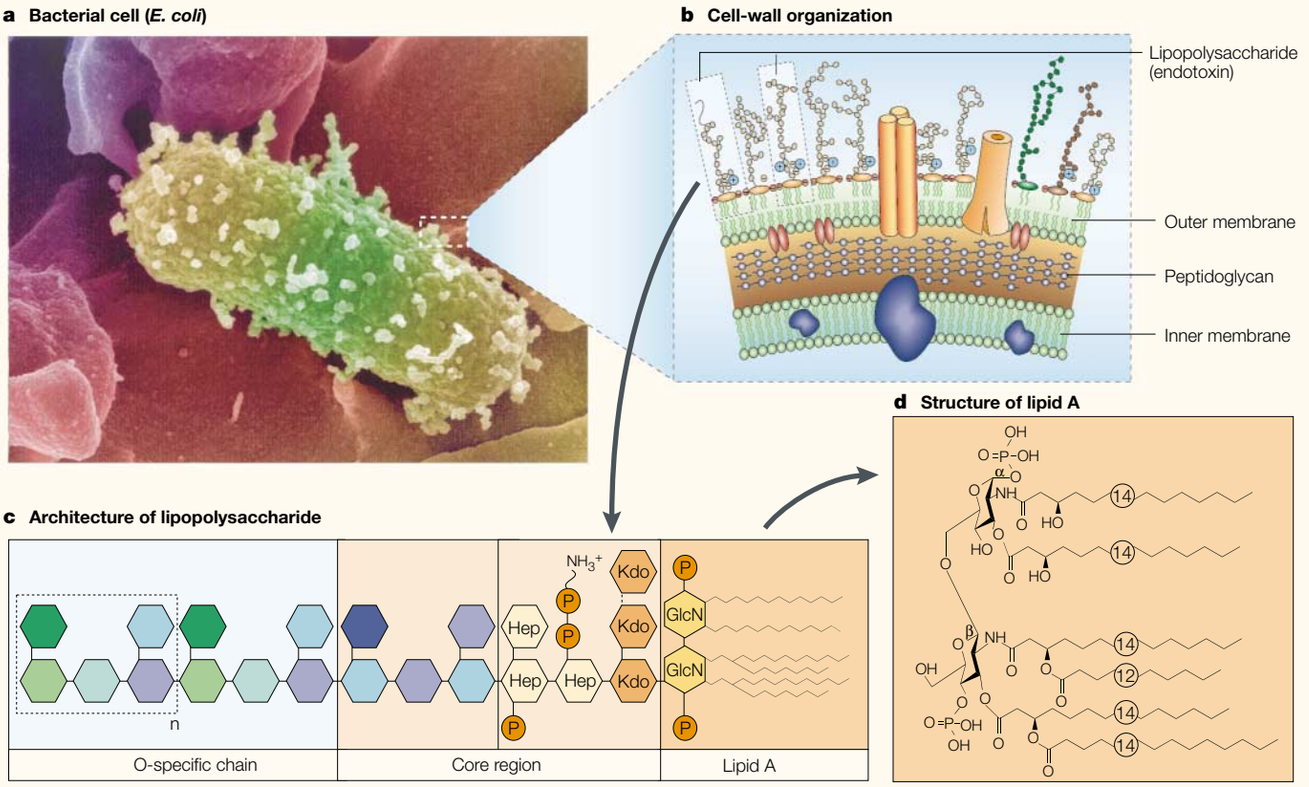


Figure 2 | **A Gram-negative bacterium.** Electron micrograph of *Escherichia coli* (**a**), together with a schematic representation of the location of lipopolysaccharide (LPS; endotoxin) in the bacterial cell wall (**b**) and the architecture of LPS (**c**). Also shown is the primary structure of the toxic centre of LPS, the lipid A component (**d**). The electron micrograph was kindly provided by M. Rhode, German Research Centre for Biotechnology, Braunschweig, Germany. GlcN, D-glucosamine; Hep, L-glycero-D-manno-heptose; Kdo, 2-keto-3-deoxy-octulosonic acid; P, phosphate.

that were discovered from serology (FIG. 2). Its purification and structural resolution required many years of effort. The development of suitable extraction procedures was a crucial technical leap in the chemical characterization of endotoxin, and both the trichloroacetic acid method of André Boivin (1895–1949) and the phenol–water procedure of Otto Lüderitz and Otto Westphal were used widely. Because of the presence of polysaccharide and lipid components, Lüderitz and Westphal designated their largely protein-free product as lipopolysaccharide (LPS), a term that had been adopted previously[18]. With these purified preparations at hand, the chemical characterization of endotoxin made rapid progress, culminating in the complete structural elucidation of certain LPS species, as well as their genetic determination and biosynthesis[19]. A particular challenge was the structural analysis of the lipid component, termed lipid A, to which several laboratories, including one of our own (E.Th.R.), contributed. Lipid A had received particular attention, as several lines of evidence indicated that it had the toxic and pyrogenic properties of endotoxin[15].

Chemical analyses showed that lipid A is an unusual glycophospholipid, having unique structural features. For example, lipid A of *Escherichia coli* LPS consists of a 1,4′-bisphosphorylated β1,6-linked D-glucosamine (D-GlcN) or a Glc2,3N disaccharide, which carries four residues of (R)-3-hydroxytetradecanoic acid at positions 2, 3, 2′ and 3′, two of which are acylated at the 3-hydroxy group by dodecanoic acid (2′) and tetradecanoic acid (2′). The hydroxy group at carbon 4 is free and that at 6′ is the attachment site for the polysaccharide component. Lipid A molecules derived from other Gram-negative bacteria follow the same architectural principle, but might differ in structural details (FIG. 2). In 1985, Shoichi Kusumoto and Tetsuo Shiba synthesized *E. coli* lipid A, thereby confirming the structure that had been deduced by analytical procedures. Furthermore, fully synthetic lipid A had, in the same doses, an identical degree of endotoxic activity as its bacterial counterpart[20]. So, an important disease-causing toxin of Gram-negative bacteria had been identified finally and was found to be a molecule of ~1,200 Da.

The chemical synthesis and biological analysis of partial structures, derivatives and analogues of lipid A then enabled structure–activity relationships to be established. It was surprising that none of the synthetic molecules had stronger endotoxic effects than *E. coli* lipid A, but rather, any modification of the structure of *E. coli* lipid A yielded products of lower endotoxicity. So, lipid A was 100-fold more toxic than monophosphoryl lipid A (MPL), and a lipid A partial structure (lacking the two secondary acyl groups) was completely devoid of toxicity in the human system. It proved, however, to be a potent endotoxin antagonist. Obviously, endotoxic activity is not dependent on a single lipid-A constituent (toxophore group), but is dependent on a defined conformation (endotoxic conformation) that is determined by unique features of the primary structure, including steric factors, negative charge and hydrophobic domains. Presumably, these features combine to give a molecule with a distinct shape, which, in turn, causes the formation of larger three-dimensional aggregates[21]. It is not clear whether monomeric LPS (lipid-A) molecules or aggregates of LPS facilitate interaction

with the host, thereby leading to endotoxic activity. Recent physical studies of Ulrich Seydel show that toxic (hexa-acyl) lipid A adopts a cubic conformation, with individual molecules having a conical shape, whereas non-toxic tetra-acyl molecules form lamellar structures, with individual molecules having a cylindrical shape[21]. So far, neither endotoxin nor lipid A has been crystallized. However, co-crystallization of LPS (together with the outer membrane iron-transport protein FhuA) and X-ray analysis at 2.5-Å resolution were achieved in 1998 by Welte and colleagues[22].

Pfeiffer himself ascribed endotoxic properties to Gram-positive bacteria, which today are known to be incapable of synthesizing LPS. It is well known that molecular constituents of Gram-positive bacteria such as *Streptococci* or *Staphylococci* cause disease and death. Therefore, poisons other than endotoxin are obviously present in bacteria, and today, in addition to proteinaceous exotoxins, several other bacterial components are known to have endotoxin-like biological effects in mammals. These include lipopeptides, peptidoglycan partial structures, lipoteichoic acid, double-stranded (ds)RNA and unmethylated DNA fragments (specifically, oligomers containing CpG dinucleotides in the correct sequence context). Each has its own story[23–26]. Although they are somewhat weaker than endotoxin in their biological potency, these molecules, in addition to other poisons, such as superantigens and pore-forming toxins, certainly contribute to the overall toxic potential of bacteria, partly by enhancing the effects of endotoxin in a synergistic manner. As it turns out, all of the molecular components named above are detected by paralogous members of a single family of host receptor proteins.

Biological definition of endotoxin

It has long been known that substances that produce a biological effect at an extremely dilute concentration often function by interacting with specific, high-affinity receptors, which are linked generally to a signal-amplification system. Therefore, the existence of an 'endotoxin receptor' was suspected from the earliest days after the chemical characterization of LPS. In view of the similar effects that are mediated by LPS and many other microbial components, it was logical to assume that a family of receptors might recognize microbial components, and to hope that elucidation of the LPS receptor might open the door to advancing our understanding of microbial pathogenesis.

The receptor — or receptors — that are responsible for the recognition of LPS would, in effect, define LPS as far as the host is concerned. Whatever the structure and conformation of LPS, a receptor agonist would be sensed as LPS, whereas a receptor antagonist would block the LPS response. The receptor, if it could be found, would be the gateway to all of the effects on an individual that arise as a result of exposure to LPS, both beneficial and detrimental.

The solitary nature of the LPS signalling pathway (and by implication, of the LPS receptor) was revealed with great clarity in 1965 by a spontaneous mutation that had become fixed in the C3H/HeJ substrain of C3H mice[27]. This mutation, affecting a single locus that was later named *Lps*[28,29], abolished all responses to LPS. So, however complex is the association between LPS and host proteins, and however complex the LPS signalling pathway might be, a single protein seemed to be required for all responses to LPS. This fact was strongly in favour of the existence of a single receptor for LPS.

Later, a second spontaneous mutation that abolished LPS responses was identified[30]. Occurring in C57BL/10ScCr animals, the mutation was shown to be allelic with the mutation in C3H/HeJ mice, because F_1 hybrid animals produced by crossing C3H/HeJ mice with C57BL/10ScCr mice were completely unresponsive to LPS (similar to the parents from which they were derived). However, the progeny that resulted from the outcross of either LPS-resistant strain to the wild-type strain were, at least partially, LPS sensitive[31].

Macrophages and the host response

Although LPS might have been thought to be toxic to many tissues and cells of the host, *in vitro* studies provided no support for this view. Somatic cells grown in culture are, for the most part, quite indifferent to LPS. Moreover, during the 1970s, definitive evidence showed that macrophages were of primary importance in the recognition of LPS[32]. More globally, haematopoietic precursors were required absolutely to support LPS toxicity[33]. Furthermore, most classes of the subphylum *Vertebrata* were remarkably resistant to LPS. Although birds were LPS sensitive *in embryo*[34], only mammals were markedly sensitive at post-developmental stages[35]. These observations began to confirm the view that LPS was a poison only by the 'choice' of the host itself. Its mode of cellular activation must be specific — otherwise, how could it fail to elicit responses from some cells and not others in a given species, and also, show such marked inter-species differences in toxicity? It was detected by cells with immune function, particularly myeloid cells, but also, in some species, by lymphoid cells. It evoked a powerful immune response. If the response was localized, and triggered by a minute inoculum of Gram-negative bacteria, it might be beneficial. If the response was systemic, and triggered by a large inoculum of Gram-negative bacteria, it might be lethal. But, direct experiments were required to test this hypothesis. The C3H/HeJ mouse again had an essential role in these experiments, although its mutational defect had yet to be identified.

The poison–protection dichotomy

So, is sensitivity to LPS a good thing or a bad thing? One might assume that it is bad, because LPS is one of the main inducers of shock in sepsis. The path of investigation described in this article established it as such. But, if sensing LPS was a bad thing, why would mammals have retained a system for its detection? A clear purpose of sensing LPS might be the timely detection of small numbers of infectious organisms, and the mobilization of an immune response to contain them.

The non-specific 'immunostimulatory' role of LPS was well described in the 1960s, when it was shown that LPS pre-treatment could protect animals against subsequent challenge with diverse pathogens (reviewed by Berger in REF. 36) and that, moreover, LPS had an adjuvant effect in the induction of adaptive immune responses (reviewed by Neter in REF. 37). However, such effects might prove to be exceptionally complex, given the complexity of the LPS response itself. The observations did not address the question of whether a response to LPS was required for the effective handling of a microbial challenge.

In the late 1970s, the protective potential of sensing LPS was analysed by infecting animals that were, in effect, 'blind' to endotoxin. These were, of course, C3H/HeJ mice, and the agent that was used to probe the importance of LPS sensing was *Salmonella typhimurium*. When administered by an intraperitoneal route, *S. typhimurium* was lethal to LPS-resistant animals at a far lower dose than it was to animals that could sense LPS normally[38,39]. In subsequent reports, the *Lps*^{d} allele of C3H/HeJ mice was shown to impair the effective containment of *E. coli* infections of the urinary tract[40], of intraperitoneally administered *Neisseria meningitidis*[41], of subcutaneous inoculation of *Francisella tularensis*[42] and perhaps of other Gram-negative organisms, in that C3H/HeJ mice were more prone to the spontaneous development of otitis media (infection of the middle chamber of the ear)[43].

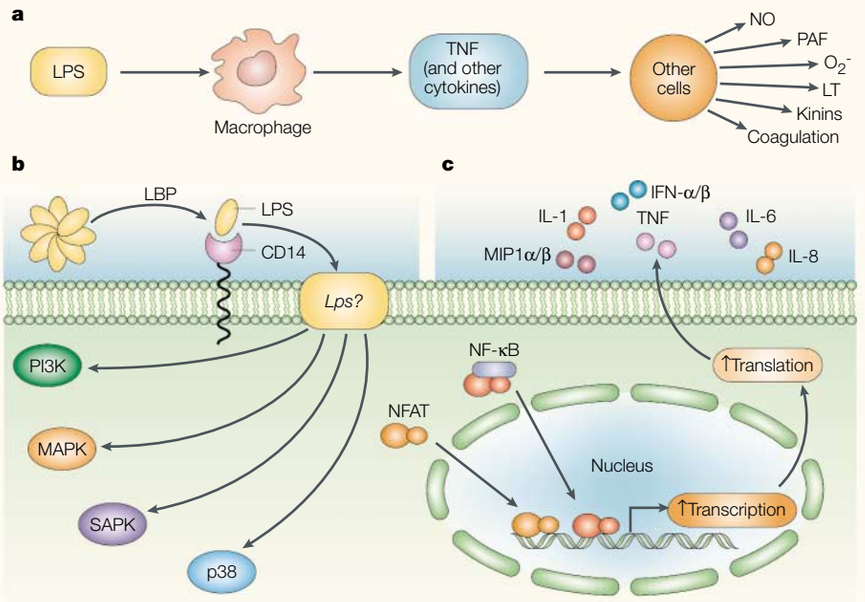


Figure 3 | **LPS signalling.** The recognition that macrophages are of central importance to the lethal effects of lipopolysaccharide (LPS), and the subsequent realization that tumour-necrosis factor (TNF) is a soluble mediator of LPS toxicity (**a**), allowed detailed biochemical analysis of the events that follow LPS stimulation. By 1990, two of the extracellular components of the LPS-sensing pathway — LPS-binding protein (LBP), which binds plasma LPS and conveys it to the surface of cells in a bioactive form, and CD14 — had been identified (**b**), as had the main transcription factor for induction of the gene encoding TNF (nuclear factor-κB, NF-κB) (**c**) and several protein kinases that are activated by LPS (**b**). It had been established also that the synthesis of TNF is subject to translational regulation. However, the transmembrane signalling component of the receptor remained unknown. The *Lps* locus — which is defective in C3H/HeJ mice — was believed to encode this component. IFN, interferon; IL, interleukin; LT, lymphotoxin; MAPK, mitogen-activated protein kinase; MIP1, macrophage inflammatory protein 1; NFAT, nuclear factor of activated T cells; NO, nitric oxide; PAF, platelet-activating factor; PI3K, phosphatidylinositol 3-kinase; SAPK, stress-activated protein kinase.

The *Lps* mutation of C3H/HeJ mice was, therefore, clearly deleterious, and it had survived as a laboratory artefact by establishing itself in the population. And LPS sensing, whatever risks it might carry, was an important part of the immediate (innate) immune response.

TNF mediates LPS responses

The essential role of macrophages in the mediation of LPS toxicity indicated that they must either produce an endogenous toxin, or act directly, to cause injury to the host once exposed to LPS. Mouse TNF was purified to homogeneity originally by one of us (B.B.) in 1985 from the conditioned medium of an LPS-induced macrophage cell line[44]. Because the protein caused a range of effects in mice that seemed to be similar to those evoked by LPS itself — for example, fever, diarrhoea, shock and death — the possibility that TNF might be an endogenous mediator of endotoxicity was entertained. Accordingly, mice were passively immunized against TNF, and then challenged with LPS. Antibody-mediated blockade of TNF caused a highly significant reduction in LPS toxicity[45], establishing for the first time that a cytokine mediator could confer the lethal effects of LPS. Therefore, TNF activity became a useful endpoint with which to monitor the effects of LPS.

TNF — similar to the LPS that induced it — had both harmful and protective qualities. Although capable of causing shock, it was required also for the effective containment of certain infections — notably listeriosis[46] and mycobacterial infection[47]. As for LPS, small doses of TNF could induce protection against subsequent infectious challenge with diverse organisms[48–50]. The degree of LPS resistance resulting from passive immunization against TNF[45] was modest compared with the degree of resistance imparted by homozygosity for the Lps^d allele[27,51]. Later, it was shown that other cytokines also contribute to the septic syndrome (the large number of physiological derangements that occur during severe infection)[52,53]. However, all of the effects of LPS seemed to be channelled through a single biochemical pathway, originally revealed by mutations at the *Lps* locus. To identify *Lps* would be tantamount to understanding LPS signalling and, perhaps, to understanding signalling by many other microbial products with similar effects. A great deal of effort was devoted to studying LPS signalling in many laboratories. Some workers focused on *Lps* itself, whereas others approached the problem from other angles.

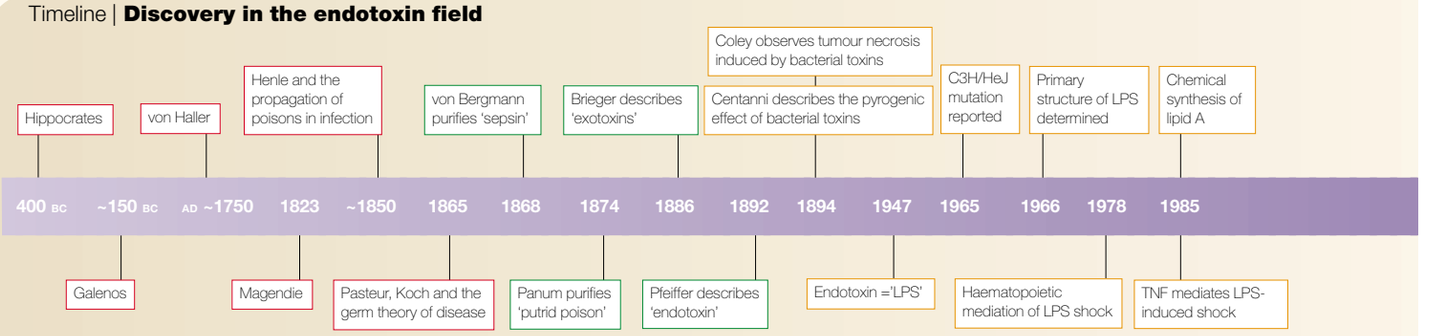
The core pathway

One of the most fruitful approaches to the study of LPS signalling involved the identification of a plasma protein, produced by the liver, that could bind LPS and that seemed to enhance the sensitivity of mononuclear cells to LPS *in vitro*[54–57]. This protein, LPS-binding protein (LBP), was produced constitutively, but in greater amounts after LPS challenge. Concomitantly, it was shown that CD14, a receptor that was anchored to the plasma membrane by a glycosylphosphatidylinositol modification, was essential for LPS sensing[58]. LBP and CD14 were, in fact, the first biologically relevant receptors for LPS to be identified. Yet, there was no clear means by which they could activate the cell, because no membrane-spanning chain had been identified. In terms of TNF production, LPS signalling events were shown, in 1990, to involve the activation of nuclear factor-κB (NF-κB)[59]. Also, LPS was found to cause the activation of numerous protein kinases, including p38 (REF. 60), stress-activated protein kinase (SAPK)[61] and phosphatidylinositol 3-kinase[62,63], in the cell. But, the earliest signalling events remained unclear. The question was a vexing one, because almost all inflammatory stimuli of microbial origin seemed to have qualitatively similar effects, yet the sensing mechanism was, in all cases, unknown (FIG. 3).

A promise fulfilled

The nature of the LPS-sensing pathway was clarified suddenly by the positional cloning of *Lps* — the end result of a five-year endeavour that fully engaged one of our laboratories (B.B.) and that was completed in 1998 (REFS 64,65). C3H/HeJ mice were shown to have a point mutation that modified a stringently conserved residue in the cytoplasmic domain of Toll-like receptor 4 (TLR4). C57BL/10ScCr mice were shown to lack TLR4 entirely. Previously an orphan receptor, a function for TLR4 had been discovered. The extensive studies carried out on C3H/HeJ mice over the years gave credence to the notion that TLR4 was an essential signalling component of the LPS receptor and indicated that its role was a highly specific one. Moreover, the discovery indicated immediately how mammalian innate immune sensing might operate in relation to other microbes and other inducing

Timeline | **Discovery in the endotoxin field**



The individuals who are responsible for particular discoveries are denoted before 1900; the events themselves are depicted after 1900. The post-microbial era began with the seminal discoveries of Koch and Pasteur (1865), and the post-genomic era is assigned to the years after 2000. Four 'phases' of discovery might be roughly circumscribed: the recognition that infection is 'poisonous' (red); the search for specific poisons, culminating in the identification of endotoxin (green); the chemical and biological characterization of endotoxin (orange); and finally, the identification of the endotoxin receptor and recognition of commonality in microbial sensing (purple). ds, double-stranded; LBP, LPS-binding protein; LP, lipopeptide; LPS, lipopolysaccharide; TLR, Toll-like receptor; TNF, tumour-necrosis factor; PG, peptidoglycan.

molecules. To understand why, it is necessary to understand the history of Toll, the prototype of the family.

Toll, a protein with a single transmembrane domain that is expressed in the *Drosophila* embryo and by cells of the fat body of adults, was identified through an entirely separate forward genetic inquiry into dorsoventral patterning. This work was carried out in the 1980s by Anderson and Nusslein-Volhard[66], and it contributed to the receipt of the 1995 Nobel Prize in Physiology and Medicine by Nusslein-Volhard. Toll was activated by the end product of a proteolytic cascade (Spätzle), and signalled by way of a serine kinase (Pelle) to activate an NF-κB-family member (Dorsal). The dual nature of Toll became apparent in 1996, when Lemaitre *et al.*[67], seizing on the observation that the promoters of genes encoding antimicrobial peptides had NF-κB motifs[68,69], showed that Toll was required for the response of *Drosophila* to fungal infection, which involved production of the antimicrobial peptide drosomycin.

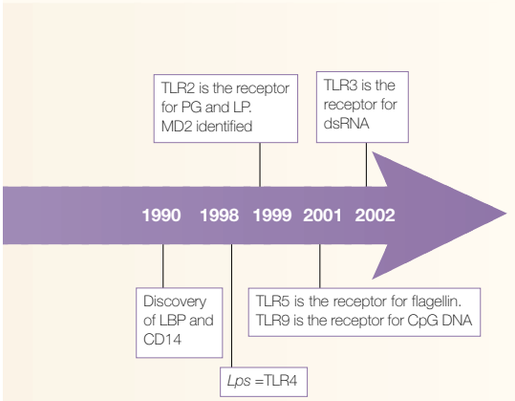
The discovery of the mammalian TLRs was preceded by the observation in 1991 (REF. 70) that the cytoplasmic domain of the interleukin-1 (IL-1) receptor was homologous to the cytoplasmic domain of Toll. The first mammalian TLR to be cloned was TLR1, which was identified as a homologue of *Drosophila* Toll in 1994 (REF. 71). Designated TIL (Toll/IL-1 receptor-like) and mapped to human chromosome 4 by Taguchi *et al.* early in 1996 (REF. 72), this protein was suspected to have a developmental function, as the immune function of Toll had not been established yet. Other TLRs were cloned in turn, each identified by searches for expressed sequence tags. However, their function had remained obscure. Transfection-based analysis of TLR function supported a role in NF-κB activation[73], but gave no clue as to the ligand that might activate any of the TLRs, whether endogenous or exogenous; nor did it prove that a ligand existed at all. For some time after TLR4 was shown to be required for LPS sensing, it was proposed that an intermediate proteolytic step must occur, as in the *Drosophila* model. The possibility is still discussed[74], although genetic[75,76] and binding[77] data indicate that there is direct contact between LPS and TLR4.

By the time that TLR4 was shown to be required for LPS signal transduction, five TLR paralogues were known to be encoded in the mammalian genome (ten human representatives and nine mouse proteins are now known to exist)[78–83]. It was reasonable to believe that each member of the family might recognize a restricted collection of microbial inducers, and indeed, to propose that these sensors might collectively sense much of the microbial world. Akira and colleagues took a leading role in testing this hypothesis, and by means of gene targeting, they determined the main microbial specificities of several TLRs, including TLR2 (REF. 84), TLR6 (REF. 85) and TLR9 (REF. 86). Also, they established the essential role of MyD88 as a transducer of signals initiated by diverse TLRs[86–88] and by the IL-1 and IL-18 receptors[89]. It is believed generally that the highly homologous TLRs TLR1 and TLR6 form heterodimeric complexes with TLR2 (REF. 90), and further, it has been shown that specificity for di-acyl versus tri-acyl lipopeptides is conferred by the specific combination of TLRs: TLR1–TLR2 heterodimers are specific for the tri-acyl congener[91]. TLR5 is required for the recognition of flagellin[92], and TLR3 is required for the recognition of dsRNA[93]. TLR7 detects imiquimod[94], a small antiviral drug with agonist activity. However, the microbial specificity of some of the TLRs (including TLR7, TLR8 and TLR10) remains undetermined to this day, and the full repertoire of specificities might emerge only with time.

The future

This account indicates that our current understanding of innate immune sensing might be traced to the early days of microbial pathogenesis, and to endotoxin, an important mediator of the damage that Gram-negative bacteria cause (TIMELINE). The identification of endotoxin as a definable chemical species was a tremendous milestone in the quest to understand how microbes create disease. The identification of soluble, host-derived mediators of toxicity — and the understanding that toxicity and protection were not readily separable from one another — was a second advance. The identification of the LPS receptor itself has closed a third chapter in the story, and it has fulfilled the promise that many microbial toxins share a mode of action that is similar to that of endotoxin.

What, then, might the next chapters hold? The microbial sensors that both harm and protect us have not been characterized fully. Some seem to reside in cells, others at the surface[95]. It is probable that the full complement of proteins that comprise these sensors has yet to be discovered, although TLRs lie at the core of the transduction mechanism. Quick on the heels of its identification as the LPS transducer, Shimazu *et al.* showed that TLR4 associates with a small protein known as MD2 (REFS 75,96), and Miyake and colleagues, as well as others, later proved definitively that this interaction is essential for LPS signalling[97,98]. It is clear that there is much structural and pharmacological work to be done. In the end, we would like to 'tame' the receptors, to trigger a response that is just right for the situation.



Both in cell culture and in whole animals, a primary challenge with LPS causes insensitivity to a secondary challenge, lasting for hours to days after the primary challenge was administered. This phenomenon, known as 'endotoxin tolerance', might be viewed as nature's attempt to mitigate the ferocity of the innate immune response, which ironically, is capable of killing the host in its zeal to defend it. We do not understand yet how endotoxin tolerance operates, nor, generally speaking, how the innate immune response is tuned to a given situation. We know but little of how the innate response activates the adaptive response, although it has been clear for many decades that an interplay does occur, to the extent that individual cytokines derived from macrophages are crucial for an effective adaptive response to develop. And it has been known for many decades that the inflammatory maelstrom brought about by microbes encourages a strong adaptive immune response.

These challenges call for further investigation at many levels. The marriage of 'chemical' and 'biological' definitions of Pfeiffer's 'endotoxin' might one day find union in the determination of a three-dimensional structure, revealing the contacts that are believed to occur between LPS and TLR4 and accessory molecules — the point at which the fire of sepsis is ignited.

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Online links

DATABASES

The following terms in this article are linked online to:

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CD14 | IL-1 | IL-18 | MD2 | MyD88 | TLR1 | TLR2 | TLR3 | TLR4 | TLR5 | TLR6 | TLR7 | TLR8 | TLR9 | TNF

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