Endotoxin, Toll-like receptor 4, and the afferent limb of innate immunity

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Positional cloning work and subsequent biochemical analyses have revealed that Toll-like receptor 4 (Tlr4) transduces the lipopolysaccharide (LPS) signal, alerting the host to infection by Gram-negative bacteria. Moreover, it appears that the LPS sensing pathway is a solitary one: disruption of *Tlr4* causes complete unresponsiveness to LPS. As several Tlr family members exist in vertebrates, it appears likely that the innate immune system defends the host by recognizing a small number of structurally conserved molecules that distinguish the microbial world from tissues of the host.

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Abbreviations

IL	interleukin
LPS	lipopolysaccharide
Tlr	Toll-like receptor

Introduction

In the 19th century, the classic work of Metchnikoff and Ehrlich [1] revealed that the immune system of vertebrates has two major divisions. In common parlance, a 'specific' immune system and an 'innate' immune system are said to coexist, and although there is much interplay between them, they function independently in certain respects.

The specific immune system depends upon a phylogenetically recent mechanism for the generation of receptor diversity. Specific immunity is unique to vertebrates, and is notable for its anticipatory character, in that millions of avid receptors are fashioned to recognize any pathogen that the host might conceivably encounter.

The innate immune system is phylogenetically much older than the specific immune system, yet is no less important to metazoan life forms. Indeed, Metchnikoff first encountered innate immunity in the water flea (*Daphnia*), and in the starfish. He correctly inferred that the 'phagocytes' he witnessed in these organisms functioned to defend the host, in the case of *Daphnia* against fungal spores, and in the starfish, against the intrusion of foreign bodies (tangerine thorns).

In vertebrates, the innate immune system also has the added duty of cooperation with the specific immune system. Once activated, innate immune cells such as macrophages produce cytokines that stimulate lymphocytes and abet the development of specific immunity. There is no doubt that innate immune cells are the first to sense the invasion of pathogens. Specific immunity was built on top of the innate immune system. Without the coordinating cytokines — indeed, without antigen-presenting cells — lymphocytes do not function efficiently. Hence, the central question arises: how do cells of the innate immune system distinguish self from non-self? Or, given innate tolerance to the host, how do innate immune cells detect their microbial quarry?

It is known that innate immunity operates at a lower level of resolution than specific immunity. Macrophages make no distinction among histocompatability antigens. Hyperacute rejection notwithstanding, macrophages do not recognize xenografts from closely related species, so far as is known. They do, however, recognize protozoa, fungi and bacteria. The present review focuses upon a group of receptors now known to subserve this recognition. The best understood of these is the lipopolysaccharide (LPS) receptor, now known to be the Toll-like receptor 4 (Tlr4).

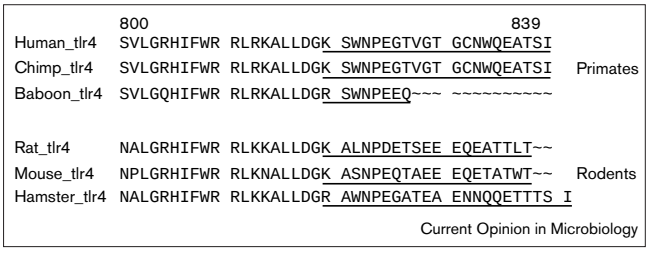
Responses to LPS

LPS is a pervasive attribute of Gram-negative bacteria. An amphiphilic molecule that is inserted into the outer leaflet of the outer membrane of Gram-negative cells, LPS has no structural homolog among multicellular organisms. Were one to design an innate immune system, LPS would surely be among its targets.

LPS has long been known to be the most toxic constituent of bacterial "endotoxin", a term first coined by Pfeiffer to denote an abundant, cell-associated product of Gram-negative bacteria that is capable of causing fever, shock, and organ injury in mammals [2]. Although many organisms, including invertebrates, respond to LPS in some fashion, violent (shock-related) reactions to LPS are a relatively new development in evolution. Birds, reptiles, amphibians, and fish do not progress to shock as a result of LPS injection [3] (though interestingly, birds appear highly sensitive to LPS during embryonic life [4]). Indeed, a number of genera of mammals (e.g. rats, mice, baboons) are relatively LPS resistant [3,5], and LPS hypersensitivity (witnessed, for example, in ungulates, humans, and rabbits) is quite haphazard among species.

LPS was once believed to act by perturbing biological membranes, and to cause tissue injury via a direct effect. The essential role of lymphoreticular cells as mediators of the LPS effect [6], and the subsequent finding that tumor necrosis factor (TNF) [7] produced by macrophages [8] was the chief mediator of endotoxic shock put an end to this idea, as it became evident that LPS worked its toxic effects by stimulating macrophages to release toxic mediators.

Figure 1



The carboxy-terminal end of the Tlr4 protein shows pronounced variability among species. Numbering refers to the human sequence. Underlined portion denotes the variable domain.

However, the mechanism of macrophage activation by LPS remained uncertain.

In 1990, the abundant glycosylphosphinositol-tethered leucine-rich protein CD14 was identified as the physical receptor for LPS on the surface of mononuclear phagocytic cells [9]. It was also observed that LBP, a plasma protein produced in the liver, acts to convey LPS to the cell surface [10–12] and may catalyze its transfer to CD14 [13–15]. A period of puzzlement then ensued, since CD14 had no cytoplasmic domain and, therefore, could not be expected to directly transduce the LPS signal. Although a number of other proteins were proposed as such, no 'co-receptor' for CD14 was identified through direct biochemical and/or cDNA cloning approaches.

LPS-resistant mice offer insight into LPS signal transduction

The solitary nature of the LPS signal transduction pathway was suggested by the discovery that mice of the strain C3H/HeJ were highly resistant to all of the biological effects of LPS [16], whereas animals of closely related substrains C3H/HeN and C3H/OuJ were noted to have normal sensitivity. LPS resistance in C3H/HeJ mice was ascribed to a single, co-dominant allele of the so-called *Lps* locus, which was mapped to chromosome 4 in 1978 [17,18]. Mice of the C57BL/10ScCr strain were also found to be profoundly resistant to LPS [19], whereas closely related animals of the C57BL/10ScSn strain were not. This resistance was ascribed to a second, recessive mutation occurring at the same locus that confers LPS resistance in C3H/HeJ mice [20]. The fact that mutation of a single gene was sufficient to block LPS signal transduction completely indicated the existence of a single biochemical pathway for LPS signaling. The fact that one — and not many — transduction pathways might exist made the *Lps* locus an appealing target for positional cloning.

Further, it was noted that mice of the C3H/HeJ strain are abnormally sensitive to infection by Gram-negative bacteria. As such, they succumb to small inocula (e.g. 1–2 organisms of *Salmonella typhimurium*) that are harmless to endotoxin-sensitive animals [21,22]. This observation must be taken to mean that LPS sensing is important to the organization of an adequate antimicrobial defense.

Invertebrate immunity involves pathways homologous to those in vertebrates

The advantages offered by *Drosophila* as system for genetic inquiry led to an important advance that superceded parallel studies in mammals (see review by Imler and Hoffmann, this issue, pp 16–22). Bereft of a specific immune system, insects and other invertebrates are entirely dependent upon innate immunity for the development of an effective defense against pathogens. Antimicrobial peptides (e.g. cecropin, attacin, and drosomycin) are the final effectors of immunity. Their production is linked to sensors that detect host invasion.

The *Drosophila Toll* locus, earlier known to be involved in dorsal-ventral patterning of the embryo, encodes a leucine-rich plasma membrane protein that was shown to mediate detection of fungal pathogens. Mutations of *Toll* (and components of its signaling pathway) lead to an immunocompomised state in *Drosophila* [23], in which fungal growth is not effectively countered. Importantly, the fungal product that initiates signaling via Toll is not yet known, and it is clear that there is no direct contact between any component of the fungus and the Toll receptor itself [24•]. On the contrary, a proteolytic cascade leading to cleavage of a pro-peptide to yield the soluble mediator Spätzle is initiated by fungal infection. At least one other member of the Toll family (seven of which are recognized in *Drosophila*) confers resistance to bacteria [25].

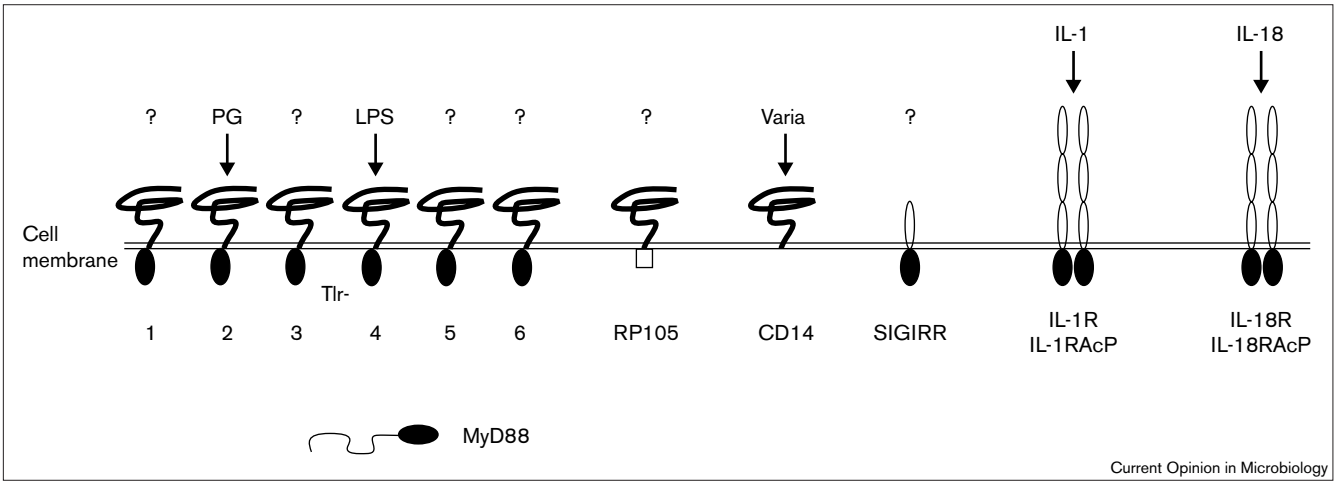
The cytoplasmic domain of each of the two interleukin (IL)-1 receptor chains was noted to be homologous to the cytoplasmic domain of Toll [26,27]. Similarly, the IL-18 receptor chains bear homology to Toll. Although IL-1 and IL-18 are undoubtedly involved in the inflammatory response in mammals, they are not homologous to Spätzle, nor are they considered to be components of a prefabricated LPS sensing pathway; rather, they are produced in response to LPS.

Indeed, the relationship between the Toll pathway and the IL-1 signaling pathway might have been considered happenstance, if not for the fact that a single human patient with a clinically significant immunodeficiency disease was found to exhibit co-resistance to IL-1 and LPS [28]. This suggested that a common signaling intermediate must serve both the IL-1 and LPS response pathways. And in turn, it might have been guessed that the IL-1 and LPS receptors were structurally similar to one another. In the event, little notice was taken of the clinical observation prior to the positional cloning of *Lps*.

Positional cloning of *Lps*

Beginning in 1994, Poltorak and co-workers began to map the mouse *Lps* locus to a high resolution. Genetic and

Figure 2



Vertebrate Tlrs and their leucine-rich relatives. Six members of the Tlr family are now known to exist in mammals and are designated Tlr1 through Tlr6. Assignment of function has been made for Tlr4 (which detects lipopolysaccharide; LPS) and Tlr2 (which detects peptidoglycan; PG). RP105 is very similar to other members of the Tlr family, but has a small cytoplasmic domain with no similarity to the Toll-like domain. CD14 is a GPI-tethered protein that is leucine rich, and perhaps distantly related to the Tlrs. Although it does not signal directly, it may convey microbial products (Varia) to other members of the Tlr family, which in turn announce their presence to the cell. SIGIRR and both chains of the IL-1 and IL-18 receptors (R), have conserved Toll-like domains, but ectodomains with homology to the immunoglobulin family of receptors. MyD88, a cytoplasmic transducer, also has a Toll-like domain, a fact which suggests that the Toll-like domain functions as a multimerization motif. IL-1RAcP, IL-1R accessory protein; IL-18RAcP, IL-18R accessory protein.

physical mapping efforts culminated in 1998 with the assignment of the locus to a genetic interval 2.6 Mb in physical size [29••]. Within this region, only a single gene was identified through high-density sequencing. This gene encoded the mouse Toll-like receptor 4 (Tlr4), an orphan receptor previously encountered as an expressed sequence tag with homology to the *Drosophila Toll* locus. Although the ligand for Tlr4 was not known, it had been shown that the protein could signal via MyD88, a cytoplasmic protein with homology to Toll, and then via the IL-1 receptor associated kinase (IRAK) and TRAF6 to activate NF-κB translocation to the nucleus [30].

Poltorak *et al*. [31••] cloned the *Tlr4* cDNA from C3H/HeJ and C3H/HeN mice, and identified a point mutation in a region corresponding to the third exon of the C3H/HeJ *Tlr4* gene, leading to the substitution of a histidine for an invariant proline residue within the cytoplasmic domain of the receptor. In C57BL/10ScCr mice (but not C57BL/10ScSn mice), the *Tlr4* locus was found to be deleted entirely (with respect to the reference genomic sequence, an interval 74723 bp has been expunged) (A Poltorak *et al*., unpublished data). As such, it became clear that mutations in the *Tlr4* gene were responsible for the lack of response of C3H/HeJ and C57BL/10ScCr mice to LPS. Hoshino *et al*. [32•] artificially deleted the *Tlr4* locus in mice and, in so doing, independently confirmed that mutational destruction of Tlr4 leads to profound unresponsiveness to LPS.

A parallel approach to the identification of the LPS signal transducer, based on transfection, was mounted by Yang *et al*. [33] and by Kirschning *et al*. [34], who both found that Tlr2 (and surprisingly, not Tlr4) was capable of transducing the LPS signal when overexpressed in 293 cells, a human embryonic kidney line that is normally insensitive to LPS. It is, by this time, quite clear that this result was the product of a system artifact, insofar as mutations of Tlr4 entirely abolish LPS signaling (leaving no room for the belief that Tlr2 is an alternate transducer), and destructive mutations of Tlr2 do not impair LPS signaling in mice [35••] or hamsters [36] (revealing that Tlr2 is not required for LPS signaling). Indeed, it now appears that Tlr2 has nothing whatever to do with LPS signaling.

In part, transfection-based analyses of Tlr function may have produced erroneous results because the cells used in these experiments were not components of the innate immune system, and as such, may have lacked essential parts of the LPS signal transduction apparatus. When Tlr4 is overexpressed in macrophages rather than 293 cells, a very different outcome is observed [37]. Augmentation of Tlr4 surface expression markedly enhances LPS sensitivity, whereas overexpression of the $Tlr4^{Lps\text{-}d}$ allele represented in C3H/HeJ mice blocks LPS signaling almost completely. Hence, Tlr4 is not only required for LPS signaling, but is also the limiting factor in LPS signaling.

Tlr4 enters into direct physical contact with LPS

It has long been known that LPS partial structures (in particular, tetra-acyl forms of LPS, which lack secondary acyl chains) show pronounced species specificity in their mode

of action. Hence, lipid A (the toxic moiety of LPS) is a powerful activator of mouse and human macrophages, whereas tetra-acyl lipid A (differing from lipid A in that two secondary acyl chains are absent from the molecule) is agonistic in mouse cells only. In human cells, tetra-acyl lipid A strongly antagonizes LPS signal transduction [38–46]. The species origin of CD14 cannot explain the different response patterns of human and murine cells [47]. As determined recently, however, the species origin of Tlr4 does dictate whether tetra-acyl lipid A will be recognized [48••]. Hence, macrophages derived from C3H/HeJ mice show no response to any LPS-like agonist as a result of the *Tlr4* mutation present in this strain. When transfected to express the common wild-type allele of Tlr4, these cells exhibit full complementation, responding to LPS, lipid A, and tetra-acyl lipid A. However, when transfected to express the common wild-type allele of human TLR4, these cells respond only to LPS and to lipid A.

Since Tlr4 'reads' the structure of an LPS congener and decides whether or not to respond to it, LPS must come to lie in extremely close proximity to Tlr4 in the course of signal transduction. In all likelihood, there is direct contact between the two molecules. The residues that differ between mouse and human Tlr4, permitting discrimination by the latter, remain to be determined.

Polymorphism at the *Tlr4* locus

A second, albeit indirect, line of evidence leading to the conclusion that LPS and Tlr4 contact one another is derived from sequence analysis, performed both within species and among them. The ectodomain of the TlR4 protein is far more variable among species than the cytoplasmic domain. Excluding the highly variable carboxyl terminus of the Tlr4 protein, the cytoplasmic domain is strongly conserved across species (Figure 1). By contrast, the ectodomain is subject to mutation along its length: far more so than would be typical for a protein-binding receptor.

Approximately 75% of the human population is homozygous for the most common structural allele of TLR4. The most common variant allele (observed at approximately 6% frequency in the Caucasian population; TLRHB — Gb:AF177766) contains a double amino acid substitution, and each substitution has been identified in humans independently, though at far lower frequency. It would seem most plausible that a rare crossover event led to the double mutation. Thereafter, the frequency of this allele must necessarily have risen. This might be taken to reflect genetic drift; however, more likely, it reflects selective pressure exerted by a microbe yet unidentified Gram-negative microbe (I Smirnova *et al*,. unpublished data).

In mice numerous structural variants of Tlr4 have been identified. The ancestry of these variants may be traced through haplotype analysis; however, correlations between structure and activity have yet to be examined.

It is likely that in humans, as in mice, mutations of TLR4 may influence susceptibility to Gram-negative infection, or the course of infection once it has been established. It is also possible, given the powerful signals that are elicited by Tlr4, that certain inflammatory diseases of humans may arise as the result of TLR4 mutations, occurring either at a germline or somatic level. It is known that certain Toll mutations exhibit gain-of-function phenotypes, and it is to be expected that constitutive activation via Tlr4 will also be encountered, although such mutations might well prove lethal to the embryo.

The function of Tlr2 and other members of the Tlr superfamily

The Tlr family of proteins is schematically illustrated in Figure 2. Six proteins (designated Tlr1 through Tlr6) with leucine-rich ectodomains and Toll-like cytoplasmic domains have been recognized and named to date, though as of this writing, three more Tlrs are plainly visible in genomic DNA; one protein (rp105) has been found to have a Toll-like ectodomain but lacks a classic Toll-like signaling domain. Five plasma membrane receptor chains are known to have Toll-like cytoplasmic domains, but lack Toll-like ectodomains. As of writing, no novel Toll-like cytoplasmic domains remain unaccounted for in the database of expressed sequence tags (dbEST) database of expressed sequence tags. This may be taken to mean that few, if any, remain to be found, or alternatively, might suggest that dbEST contains an inadequate representation of mammalian cDNAs. The truth is likely to be somewhere in between.

Henceforth, knockout work will be the greatest help in assigning function to members of the Tlr family of proteins. There is a sense that those representatives with leucine-rich ectodomains subserve pathogen recognition; however, if the *Drosophila* model is true in all respects, some may have developmental functions as well. It is now known that Tlr2 is required for the recognition of peptidoglycan: it is, as such, the muramyldipeptide (MDP) transducer (MDP being the smallest peptidoglycan unit) [35••]. It may also transduce signals from other molecules — notably lipopeptides — though surely the list of claimed activators, based on transfection data, surpasses the list of authentic activators. It may be anticipated that fungi and protozoal parasites also induce responses through Tlrs. Indeed, Tlrs may be the very 'eyes' of the innate immune system.

It is to be expected that most interactions between Tlrs and pathogen molecules will entail direct binding, as has been established for LPS and Tlr4. Although the situation is quite different in *Drosophila*, it is possible that the upstream proteolytic cascade was diverted for other uses in course of evolution. The complement cascade and the coagulation cascade — also triggered by pathogens — may be the modern descendents of the primitive ancestral pathway leading to activation of primordial Toll family members.

Conclusions

An intensive search for the endotoxin signal transducer defective in C3H/HeJ mice revealed that Tlr4 functions as the sole gateway to LPS responses. Tlr4 is heir to an ancient family of receptors that sense infection. The prototypic member of this family, Toll, defends *Drosophila* against fungal infection, but does so without ever "seeing"the infectious agent itself. In mammals, mutations of Tlr4 forbid LPS signaling, and overexpression of Tlr4 vastly augments LPS signaling. Moreover, Tlr4 has direct contact with LPS. In all probability, other Tlr molecules act in a similar fashion. It is difficult to overstate the importance of these receptors, which represent direct interfaces between cells of the innate immune system and elements of the microbial world. Loss-of-function mutations in Tlrs may lead to selective immunocompromise in humans, and gain-of-function mutations may cause exaggerated or spontaneous inflammatory responses. We may look with anticipation to the development of drugs that selectively target Tlrs, effectively mitigating the worst consequences of microbial infections.

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