

INNATE IMMUNE SENSING AND THE TOLL-LIKE RECEPTORS

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Placed in the path of portal blood draining the gut and other enteric structures, the liver is often the first organ to encounter microbes or their molecular constituents, and has developed an immune function that coexists with its metabolic function. Immunity is subserved largely by Kupffer cells lining the hepatic sinusoids. As such, it is innate immunity rather than acquired immunity that dominates in the detection of foreign molecules. Among inducers of innate immune responses, lipopolysaccharide (LPS, or endotoxin; a product of gram-negative organisms), lipopeptides, peptidoglycan, unmethylated DNA, zymosan, and many other agents have been described. LPS is perhaps the most powerful of these. The mechanism by which it is detected long eluded understanding but has come into sharp focus with the identification of Toll-like receptor 4 as the plasma membrane protein that binds LPS and mediates all LPS responses. This receptor, and others like it, are presumed to be the principal exponents of innate immune sensing.

THE GENERAL STRATEGIES OF INNATE AND ACQUIRED IMMUNITY

Vertebrates alone possess an acquired immune system, that is, one that requires exposure to an antigen to develop its full potency. The acquired immune response takes days or weeks to become fully effective, and grows stronger with repeated exposure to the inciting antigen. It is mediated by lymphocytes, which arose only recently in metazoan evolution. Lymphocytes are collectively endowed with the extraordinary ability to generate millions of different receptor molecules through rearrangements of their own genomic DNA. As such, one lymphocyte or another is likely to react with any foreign macromolecule the host encounters. In the course of development, autoreactive clones are eliminated, and it is at this point that a distinction between self and nonself is made.

Remarkable as acquired immunity may be, it is insufficient to protect the host. So much is obvious from the fact that neutropenic individuals are severely immunocompromised, despite the continued presence of functional lymphocytes. Furthermore, the acquired immune system cannot operate without supporting cells, notably macrophages and dendritic cells, which present antigens to lymphocytes in a

recognizable molecular format. These cells also stimulate lymphocytes to divide by activating specific mitogenic receptors through the elaboration of soluble and membrane-bound ligands (cytokines). Without the assistance of myeloid cells, acquired immunity would be an impossibility.

In a sense, this is not surprising. The “newer” acquired immune system (also variously referred to as the “adaptive” or “specific” immune system) was built atop the older system of innate immunity (also called “natural” immunity). First recognized as an important protective mechanism by Metchnikoff more than 100 years ago, phagocytes are more than they appear. While the ameboid cells that patrol the body are not sentient beings, they behave with what seems a kind of intelligence. They are discriminating in their behavior, attacking only microbial invaders and tolerating cells and tissues of the host. Hence, long before the acquired immune system evolved, the innate immune system was fully capable of distinguishing self from nonself. Furthermore, though lacking the immense repertoire of receptors for which the lymphoid compartment is famous, innate immune cells nonetheless cope with an immense variety of pathogens.

How do they accomplish this? The inescapable conclusion is that innate immune cells must utilize receptors of a

very special type. These receptors must never react with healthy host tissues, but must somehow react with virtually all pathogens. The receptors must not be of numerous types: enough is known of the genome to realize that a huge array of receptor genes (comparable, for example, to the repertoire of rearranged immunoglobulins) is not present in the germline. Therefore, only a limited number of microbial determinants must be targeted by the innate immune system. These determinants might be presumed to be indispensable for the pathogens, since mutation and selection among pathogens would otherwise bring about their loss.

THE RESPONSE TO LIPOPOLYSACCHARIDE AS A PARADIGM OF INNATE IMMUNE SENSING

The receptors of innate immunity trigger what we have come to call the inflammatory response. They lead to the release of cytokine mediators that activate other innate immune cells at a distance, preparing the host for the spread of infection in the event that immediate containment is unsuccessful. This is much in evidence in the response to LPS.

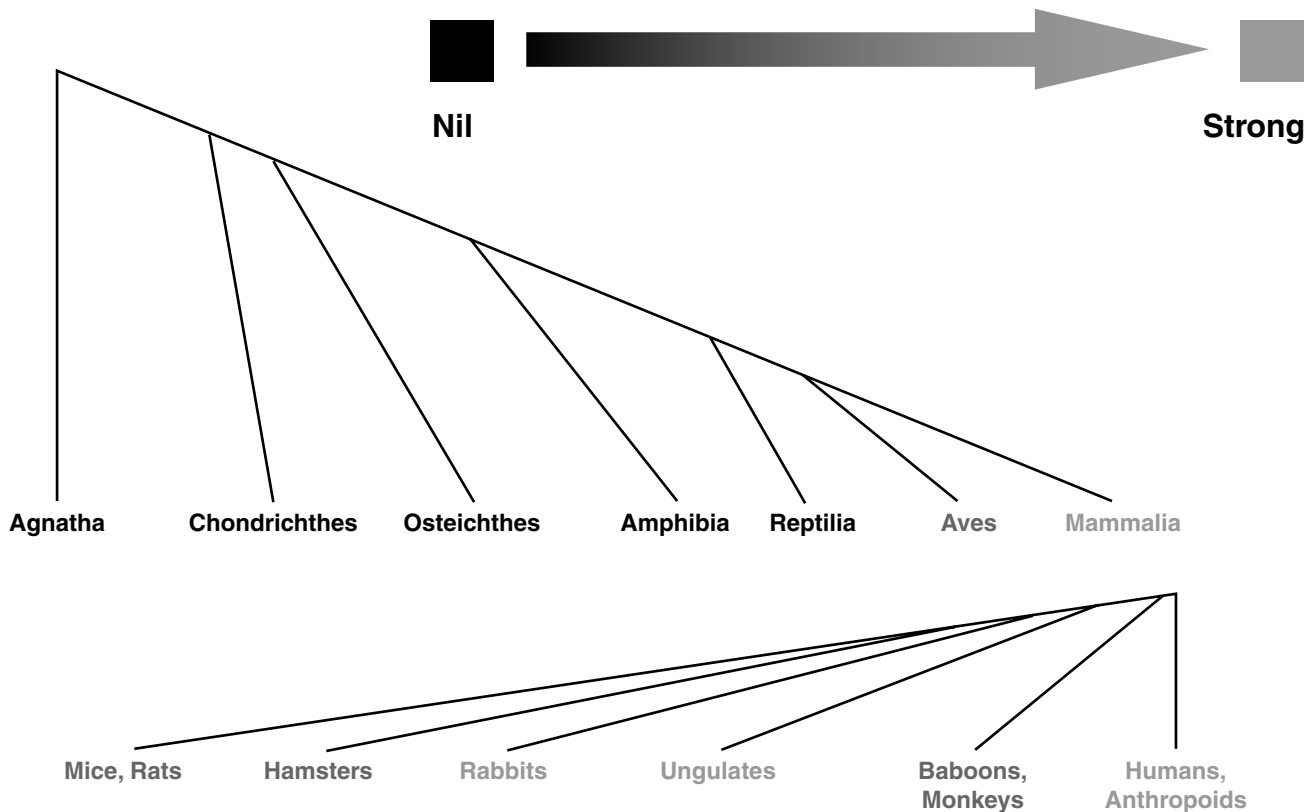


FIGURE 41.1. The phylogeny of lipopolysaccharide (LPS) responses. *Light gray* color indicates an intense response; *black* indicates no response; *shading* in between indicates responses of intermediate intensity. Only mammals show pronounced sensitivity to LPS, and among mammals, great variability is observed.

LPS fulfills the criterion of indispensability. It is present on nearly all gram-negative bacteria, and is an important structural component of the outer membrane. It has indeed become a target of the innate immune system, and macrophages react violently to it, releasing tumor necrosis factor (TNF), interleukin-1 (IL-1), and many other cytokines when they encounter it. These cytokines, if overproduced, can cause shock and death. However, at lower levels, they are known to mediate protective effects, against gram-negative organisms and also against unrelated infectious agents of gram-positive, fungal, or protozoal taxa. The fact that cross-protection occurs may indicate that the innate immune system deals with many pathogens in a similar manner. Among the end effects of cytokine release are the induction of fever, the synthesis and release of acute-phase reactants, and vasodilation with a fall in blood pressure. Interestingly, the response to LPS is phylogenetically erratic (Fig. 41.1). While some invertebrates respond to LPS in some fashion (witness the gelation response of *Limulus* amoebocyte lysates), most are quite indifferent to it, as are most vertebrates. Indeed, birds are minimally susceptible to LPS toxicity, and among mammals, a great deal of variability exists, so that even rather closely related families of *Mammalia* (e.g., the lower primates vs. the anthropoid apes) show very different patterns of response. This fact in itself could be taken to suggest that a few, critical determinants of the LPS response might exist in mammals.

There has been considerable mystery as to how LPS triggers macrophage responses. In 1990, a partial answer came to light with the finding that the LPS is immobilized on the macrophage surface by CD14 (1), a glycosylinositolphosphate-linked protein with a leucine-rich structure. It was found to be transferred to CD14 by the action of LPS binding protein (LBP) (2–5), a plasma protein produced by the liver and now known to offer protection against certain gram-negative bacteria (6). However, these findings did not reveal the mechanism by which a transmembrane signal was generated. CD14 has no cytoplasmic component, and cannot evoke such a signal.

Many attempts were made to identify a second “signaling” receptor for LPS. Direct efforts at purifying such a receptor through the use of affinity methods, or by cross-linking CD14 to other proteins on the cell surface, were fruitless. Many candidates were proposed, but none survived rigorous experimental analysis.

THE SEARCH FOR THE *Lps* GENE

The signaling component of the LPS receptor was identified through genetic studies undertaken with mice that were unresponsive to LPS. In 1965, C3H/HeJ mice were found to be resistant to the lethal effect of LPS (7). Resistance was ultimately ascribed to a spontaneous mutation, occurring at a single locus on chromosome 4 (8,9). This

locus was termed *Lps*, and the particular allele present in C3H/HeJ mice was termed *Lps^d*, to denote a defective or deficient response to LPS. The mutation conferred total or near-total insensitivity to LPS, and in future analyses of signal transduction using cytokine production as an end point, it became very clear that the LPS dose-response curve was displaced by three to four orders of magnitude in cells derived from C3H/HeJ mice. Interestingly, C3H/HeJ mice were found to be hypersusceptible to authentic gram-negative infections (10,11). Therefore, it became clear that timely detection of a gram-negative infection via the LPS signaling pathway was essential for an effective host response.

In 1978, a second mutation at the *Lps* locus was reported in an unrelated *Mus musculus* strain. C57BL/10ScCr mice were noted to be similarly unresponsive to LPS, and when crossed to C3H/HeJ mice, produced unresponsive progeny (12). However, the defect in these animals was recessive, while the C3H/HeJ defect was codominant. This implied that the mutations at *Lps* involved the same gene, but were structurally dissimilar.

Among the speculations concerning the *Lps* locus, and the critical gene it contained, were that a component of the interferon- α/β complex (also on chromosome 4) might be the gene; that a signaling protein such as protein kinase C might be encoded by it; and that it might code for a microtubule-associated protein (since taxol, a weak LPS mimetic, is known to associate with microtubules). Complementary DNA (cDNA) cloning approaches failed to identify the *Lps* gene product, though it was claimed on the basis of weak evidence that the guanosine triphosphate (GTP)-binding protein Ran/TC4 was encoded by the gene in question (13). In fact, Ran/TC4 is encoded by a gene that resides on mouse chromosome 17, and is thus entirely unrelated to the *Lps* locus.

In the end, the *Lps* mutations were identified through positional cloning efforts by Poltorak et al. (14), who refined the location of the mutation in C3H/HeJ mice on 2,093 meioses, sequenced the major part of a large (2.6 megabases) critical region, and found that only a single gene was present in the entire interval. This gene coded for the orphan receptor, Toll-like receptor 4 (Tlr4). C3H/HeJ mice were found to have a point mutation of *Tlr4*, whereas the gene was deleted entirely in C57BL/10ScCr mice (15,16).

EVOLUTIONARY ORIGINS OF TLR4

As noted earlier, the innate immune system is phylogenetically older than the acquired immune system. Moreover, it is the only immune system in invertebrate organisms. Basic studies of invertebrate immunity therefore provide a template for the understanding of immunity in higher organisms. In 1996, it was reported that the *Drosophila* Toll protein, previously known to have a developmental function in

the embryo (17,18), was required for an effective response to fungal infection in the adult fly (19). The existence of such a function was guessed from the fact that fungal growth is ordinarily controlled by drosomycin, an antifungal protein under the control of a promoter driven by members of the Rel family of transcription factors. Two such factors, Dif and Dorsal, are activated as a result of Toll ligation (20), which occurs in embryo when the prohormone Spätzle is cleaved to its mature form by the proteolytic enzyme Snake.

Mutational studies demonstrated that viable Toll-defective flies (bearing loss-of-function mutations of Toll) were susceptible to infection by the fungus *Aspergillus fumigatus*. Similarly, impairment of signaling intermediates (Pelle, Tube, or Dif) would prevent an effective response to fungal infection. The entire axis of the innate immune response to fungus was thus solved in a very short period of time. At least one other member of the Toll family in *Drosophila* has subsequently been shown to mediate resistant to bacterial infection (21). It is noteworthy that these responses occur in the *Drosophila* fat body, the functional equivalent of the vertebrate liver.

MAMMALIAN TLRs AND THEIR SPECIFICITY

It was not obvious that this same system actually applied in vertebrates, which have diverged from *Drosophila* for approximately 500 million years. It was known that homologues of *Drosophila* Toll were present in mammals, as representatives of the family had been found among expressed sequence tag (EST) databases, and on this basis five Tlr cDNAs were cloned before the function of any Tlr was assigned (22–24). Moreover, ligation of at least one of the Tlrs (Tlr4) could cause NFκB translocation in transfected cell lines (23). But the true ligands of each Tlr could not be identified through transfection studies, and all remained orphan receptors. Indeed, a concerted search for an LPS transducer among known members of the Toll-like receptor family in vertebrates ended with embarrassment, in that it was incorrectly claimed that Tlr2 was responsible for such a function, whereas Tlr4 was not (25,26). This work caused considerable confusion in the field during its earliest days.

The fact that Tlr4 (and not Tlr2) was responsible for LPS signal transduction was underscored by the knockout of *Tlr2* in mice. Animals lacking *Tlr2* were entirely normal in their response to LPS. Notably, however, they did not respond to muramyl dipeptide (the smallest unit of peptidoglycan) (27) or to certain bacterial lipopeptides (28). It was also pointed out that *Tlr4* defects create resistance to lipoteichoic acid (*Tlr2* defects do not) (27). Hence, while the function of most Tlrs remains undeciphered, the two Tlrs for which at least some functions have been assigned present a picture in which oligospecific reactivity to microbial products is the rule.

At present writing, nine mammalian Tlr cDNAs have been cloned (28a). Two subgroups of Tlrs are apparent from analyses of sequence alignments. All have a leucine-rich ectodomain and display varying numbers of leucine-rich repeat motifs. All have well-conserved Toll-like domains on the cytoplasmic side. All but Tlr2 and Tlr4 remain orphan receptors, although a common theme of innate immune response is strongly suspected.

OTHER RECEPTORS WITH TOLL-LIKE DOMAINS

In 1991, with the cloning of one subunit of the IL-1 receptor cDNA, it was recognized that strong homology existed between the *Drosophila* Toll and IL-1R cytoplasmic domains (for which reason the Toll-like domain is sometimes referred to as a Toll-IL-1R-like domain, or TIR) (29). The other chain of the IL-1R (IL-1RAcP) and both chains of the IL-18R were also found to have Toll-like domains, though both chains of both receptors have immunoglobulin-repeat ectodomains. More recently, an orphan receptor termed SIGIRR has also been identified through EST database searches, which has a well-conserved Toll-like domain and a single immunoglobulin (Ig)-repeat ectodomain (30).

The proinflammatory nature of IL-1 and IL-18 receptors is well understood. So, too, are some details of the signaling pathways that serve both of these receptors (see below). The presence of Toll-like cytoplasmic domains may be seen as an evolutionary attempt to enhance the signal elicited by an infectious organism, wherein IL-1 is elicited by the primary stimulus, and may serve to generate a greatly amplified response. Hence, a single LPS molecule might potentially cause the activation of many cells, at anatomic sites far beyond the locus of the infection.

LIPOPOLYSACCHARIDE IS A DIRECT LIGAND FOR TLR4

In *Drosophila*, the protein Spätzle is generated in response to infection, through the action of unknown protease(s) activated by pathogen molecules yet to be identified. Spätzle is the proximal ligand for Toll receptor activation, and Toll does not have direct contact with the pathogen or any of its components.

In mammals, the situation is different, in that Tlr4 (and by implication, other Tlrs) interacts directly with a component of the pathogen (in this case, LPS). The fact of direct contact was inferred from genetic complementation studies (31) in which advantage was taken of species-dependent selectivity in response to different LPS partial structures. While lipid A, the toxic center of LPS, is capable of activating both human and mouse macrophages, stimulating them to produce TNF, tetra-acyl lipid A (which lacks secondary

acyl chains) is only capable of stimulating mouse macrophages; when applied to human cells, it antagonizes activation by lipid A or intact LPS (Fig. 41.2).

By transducing an immortalized macrophage cell line derived from C3H/HeJ macrophages to express the normal mouse or human Tlr4 proteins, it was demonstrated that the species origin of Tlr4 is the sole determinant of selectivity in the response to tetra-acyl lipid A. Cells expressing human Tlr4 would not respond to tetra-acyl lipid A, though their response to intact lipid A was normal. Insofar as the Tlr4 protein “reads” the structure of lipid A and determines whether secondary acyl chains are present, it may be concluded that close physical contact between Tlr4 and the agonist must exist. Furthermore, it is clear that structural differences between different Tlr4 molecules influence reactivity with a given molecular species of LPS.

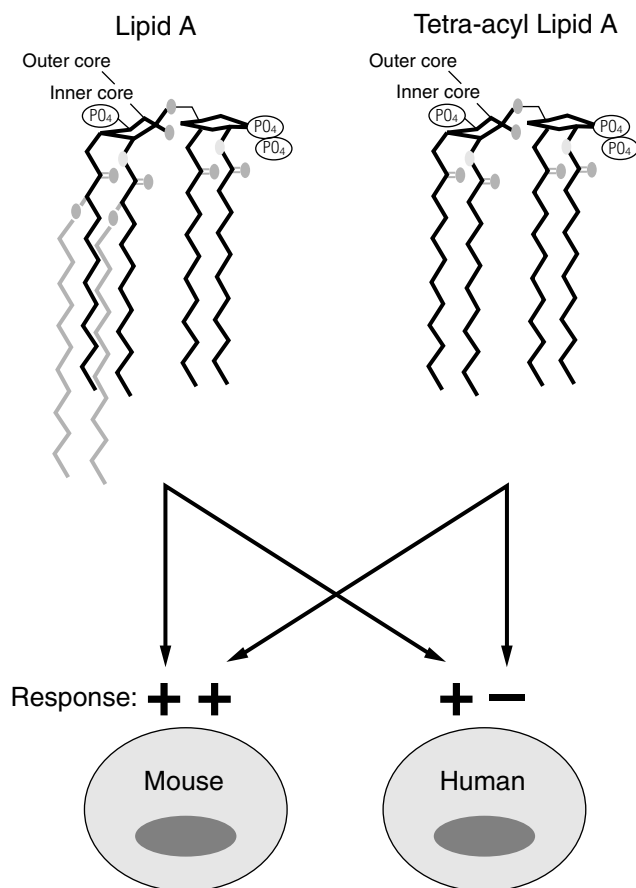


FIGURE 41.2. Interspecies differences in responses to lipopolysaccharide (LPS) partial structures. While both human and mouse macrophages show vigorous responses to lipid A, the toxic center of LPS, human macrophages fail to respond to tetra-acyl lipid A (lacking secondary acyl chains). By contrast, mouse macrophages respond strongly to tetra-acyl lipid A. This species difference was ascribed solely to the species origin of Tlr4. Hence, it is Tlr4 that interprets the structure of an LPS molecule, and differences in Tlr4 primary structure (as, for example, the difference between human and mouse Tlr4) can determine whether response to a particular LPS molecule will occur.

Similar conclusions were sustained by studies of LPS response in hamster cells transduced to express the human Tlr4 protein (32). Such differences, manifested at the level of polymorphisms occurring within a species, might influence susceptibility to specific gram-negative organisms.

It has also been shown that overexpression of Tlr4 on the macrophage surface leads to exaggerated sensitivity to LPS (approximately 30-fold lower concentrations of LPS are required to induce half-maximal secretion of TNF). It is therefore supposed that Tlr4 is a limiting factor in the transduction complex, though it is quite certain that accessory proteins are required as well. Consistent with the codominant suppressive phenotype associated with the *Tlr4*^{Lps-d} allele, overexpression of this mutant form of the protein will strongly suppress LPS signaling, essentially abolishing it in most clones examined (33).

POLYMORPHISM AT THE TLR4 LOCUS

The genes encoding human and mouse Tlr4 are placed at a considerable distance from any known neighbors, and lie in the midst of a region rich in retroviral repeats (Fig. 41.3). The human locus is in the cytogenetic interval 9q33-9q34, while the mouse locus is placed in the distal third of chromosome 4. Both human and murine forms of the gene have been sequenced to completion, and the exons of several other mammalian species have also been sequenced for the purpose of phylogenetic comparison.

Among mice, considerable variability is observed at the *Tlr4* locus. In addition to the mutations that create LPS resistance in C3H/HeJ and C57BL/10ScCr strains, a considerable amount of coding polymorphism has been observed, with 11 amino acid substitution sites observed among 35 strains, most in the ectodomain of the protein. At present, no clear phenotypic effect has been ascribed to any murine polymorphism, though potentially, such an analysis might be carried out using the same transfection system used to infer physical contact between LPS and Tlr4 (31).

Among human populations, Tlr4 also shows impressive structural variation. In Caucasians, a variant allele (TLR4-B; Gb:AF177766) in which two amino acid substitutions modify the mid-ectodomain is present at a frequency of approximately 14% in the population at large. TLR4-B is extremely rare among African Americans, and presumably arose in the European population. However, an African allele has been identified in which substitution at one of the two sites altered in the European population has occurred. This would suggest that the European allele arose upon an African background, through mutation or crossover. Moreover, among African Americans and Africans, mutation at Tlr4 is more abundant than it is among Caucasians. The difference between these populations might reflect the effects of selective pressures exerted by microbial pathogens that differed between the two geographic locations

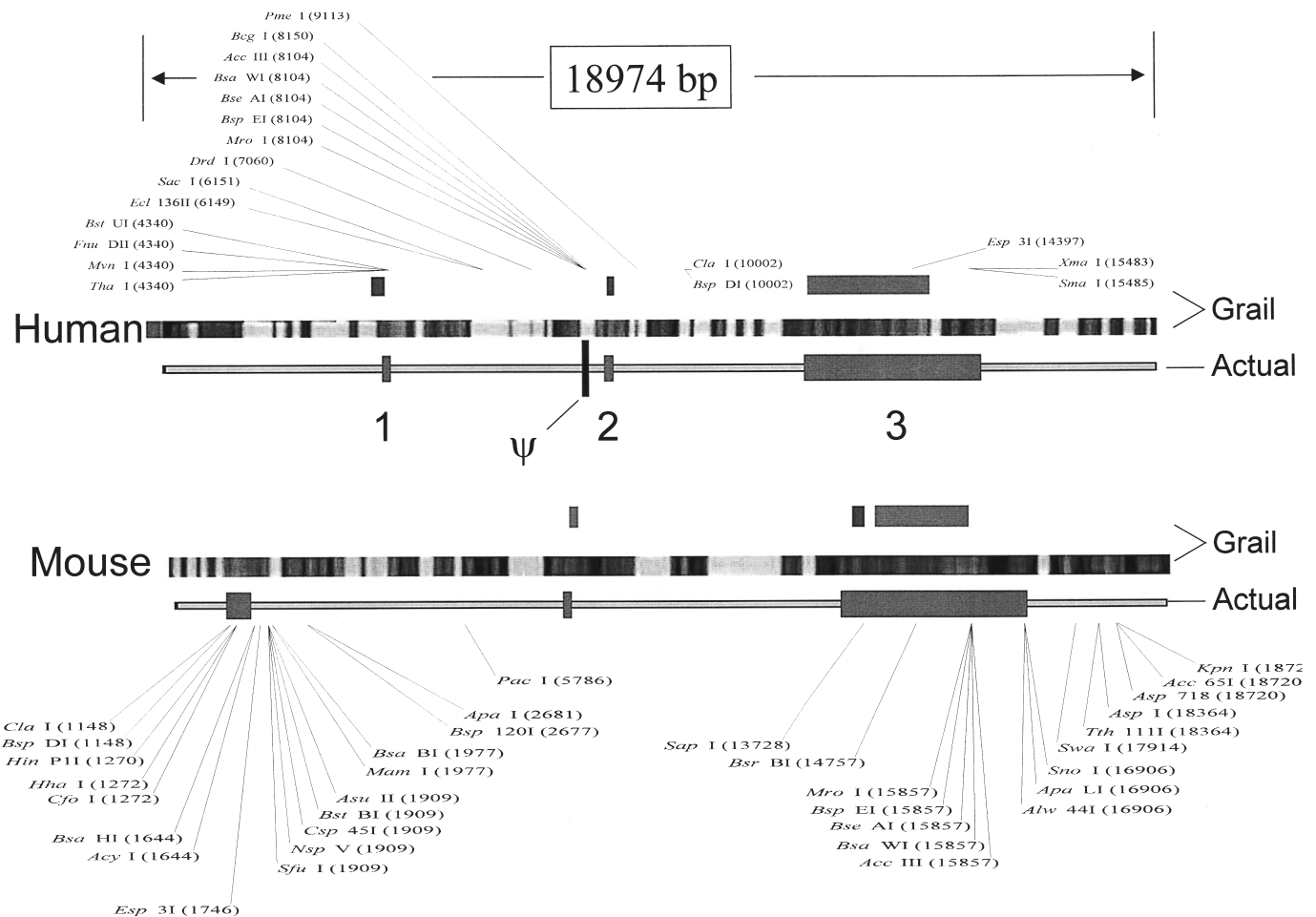


FIGURE 41.3. The genomic structure of *Tlr4* and TLR4, the human and mouse Toll-like receptor 4 genes, illustrated using the gene prediction program GRAIL. *Gray-scale image* portrays the G/C content of genomic DNA in a region spanning both genes (*darker areas* are G/C rich; *lighter areas* are A/T rich). The *light gray* regions of genomic sequence denote areas that are composed of repetitive DNA (chiefly of retroviral origin). The location of exons encoding the processed messenger RNA (mRNA) is shown as *bars* adjacent to each sequence. The actual locations of the exons are well predicted by GRAIL (*top gray bars*), which efficiently recognizes genes in anonymous DNA sequences.

(Smirnova et al., in preparation). However, in humans as in mice, phenotypic significance has yet to be assigned to the TLR4 polymorphisms that have been identified.

THE STRUCTURE OF THE LIPOPOLYSACCHARIDE SIGNALING COMPLEX

Shimazu and co-workers (34) recently reported that the small, secreted protein MD-2 may be coprecipitated from the macrophage surface together with Tlr4. Moreover, coexpression of MD-2 and Tlr4 imparts LPS responsiveness to 293 cells, which are otherwise insensitive to LPS. Although transfection-based analyses have proved misleading in the past, it is possible that MD-2 is indeed essential for LPS sig-

naling, and knockout of the MD-2 gene is eagerly awaited for this reason.

Assuming that Tlr4 requires MD-2 for effective responses to LPS, and assuming that CD14 is also essential, a tripartite protein complex may be envisioned, wherein LPS would necessarily bind to Tlr4 to elicit a signal. It cannot, at this point, be said that other proteins are not also involved. Moreover, cofactors for signaling on the cytoplasmic side have not yet been fully deciphered.

Kawai et al. (35) showed that MyD88, a cytoplasmic protein with a Toll-like domain, is required for LPS toxicity, insofar as knockout mutations greatly diminish the lethal effect of LPS. MyD88 is also required for signaling from other receptors that have a Toll-like domain (notably the IL-1 and IL-18 receptors) (36). It is presumed that this protein is recruited to Tlr4, wherein it undergoes het-

erotypic interaction with the receptor Toll-like domain to initiate a signal. Signaling may also involve phosphorylation of the receptor, though at present writing this has not been shown to occur.

Acting in conjunction with MyD88, the IL-1 receptor associated kinase (IRAK) is involved in transducing the LPS signal to the level of NF κ B (37,38). TRAF-6 is also required for this purpose, and a “bridge” between TRAF-6 and MEKK-1 is reportedly formed by ECSIT, a protein with binding affinity for TRAF-6 (39).

It must be acknowledged, however, that understanding of the LPS signal transduction cascade remains quite sketchy, as complex and as highly ramified as the pathway seems to be. For example, the events that lead to mitogen-activated protein (MAP) kinase activation, p38 activation, and phosphatidylinositol-3 (PI3) kinase activation—all of which certainly occur as a result of LPS activation (40–43)—are quite mysterious. Do other proteins engage Tlr4 directly to achieve these ends?

Mutational evidence suggests that this may be the case. In humans, a single instance of co-resistance to LPS and IL-1 has been reported in a young girl who suffered from a severe immunodeficiency (44). It would appear that this patient suffered from a global defect in Tlr signal transduction. Yet MyD88 expression and structure were normal, implying that other cofactors for signal transduction remain to be discovered.

THE ROLE OF OTHER MEMBERS OF THE TLR FAMILY

Knockout work appears to be leading the way in analysis of Tlr receptor function. Targeted mutation of the Tlr4 gene in mice confirmed the phenotype known to exist in C3H/HeJ animals and C57BL/10ScCr animals (45). Moreover, it ruled out a role for Tlr2 in LPS signal transduction (27). Importantly, however, it established that Tlr2 does play a role in peptidoglycan signaling (27) and in bacterial lipopeptide signaling (28), so that it may now be inferred that oligospecificity of Tlr receptor function is the rule. It is widely guessed that other bacterial determinants (for example, CpG dinucleotides, presented in the correct sequence context) may be sensed through other Tlrs. Such well-known inducing molecules as lipoarabinomannan (a constituent of mycobacteria) are being investigated to determine Tlr specificity. However, it is well to be mindful of the potential failure of transfection as a tool in such studies, and the gold standard for assessment of signal transducing potential must henceforth be mutational deletion of the Tlr involved.

Remarkably, CD14 shows broad specificity in its ability to engage microbial products ranging from LPS to peptidoglycan to lipopeptides. It is not yet known whether the role played by CD14 is a universal one, or whether other pro-

teins served to concentrate the signal carried by other inducers. But it is likely that the Tlrs are the principal (and perhaps the only) receptor pathway by which microbes are sensed.

CONCLUSION: THE ADMIXTURE OF INNATE AND ACQUIRED IMMUNE FUNCTIONS IN IMMUNITY AND IMMUNOPATHOLOGY

The acquired immune system came into existence in the context of the innate immune system that predated it. As lymphocytes share a common ontogenic origin with macrophages, it is not surprising that they should be endowed with Tlrs just as the latter cells are. Hence, in mice, the mitogenic effect of LPS is a direct one, and is mediated by Tlr4. Beyond the fact that innate immune and acquired immune responses are not fully separable, it might be guessed that each system might influence the other.

In the case of the innate immune system exerting an effect on the acquired immune system, there is ample evidence of such an influence. Acquired immunity could not exist without the innate immune system. Not only do the innate immune cells themselves (macrophages and dendritic cells) support an acquired immune response through the vital function of antigen presentation, but they elaborate cytokines that are indispensable for an acquired immune response to occur.

It is also clear that cooperativity occurs in the opposite direction. The output of an acquired immune response is, at one level, the production of antibody, and antibody opsonizes microbial targets so as to cause their elimination by innate immune cells. Lymphokines (most strikingly interferon- γ) can also influence the behavior of macrophages.

In proposing the occurrence of “horror autotoxicus,” Ehrlich envisioned a problem in which antitoxins (antibodies) might attack tissues of the host, leading to an autoimmune catastrophe. We know very well that this can occur. We know also that in its purest form, sepsis represents another type of autoimmune catastrophe, one in which the host is damaged by an overly exuberant innate immune response. We are left to wonder whether interplay between these two forms of immunity might exist, and specifically, whether innate immune activation via the Tlrs could contribute to various autoimmune diseases (Fig. 41.4).

It has recently been shown that antibodies against Tlr4 may have strong agonist properties if cross-linked by a secondary antibody (46). In certain autoimmune diseases, antibodies against Tlrs might actually exist. If so, it is possible that they might induce cytokine synthesis to the detriment of the host. It is also possible that certain polymorphisms of Tlrs might predispose to autoimmune disease, or that defects of signaling within the Tlr pathway could augment such disease. Awareness of the Tlrs as the principal

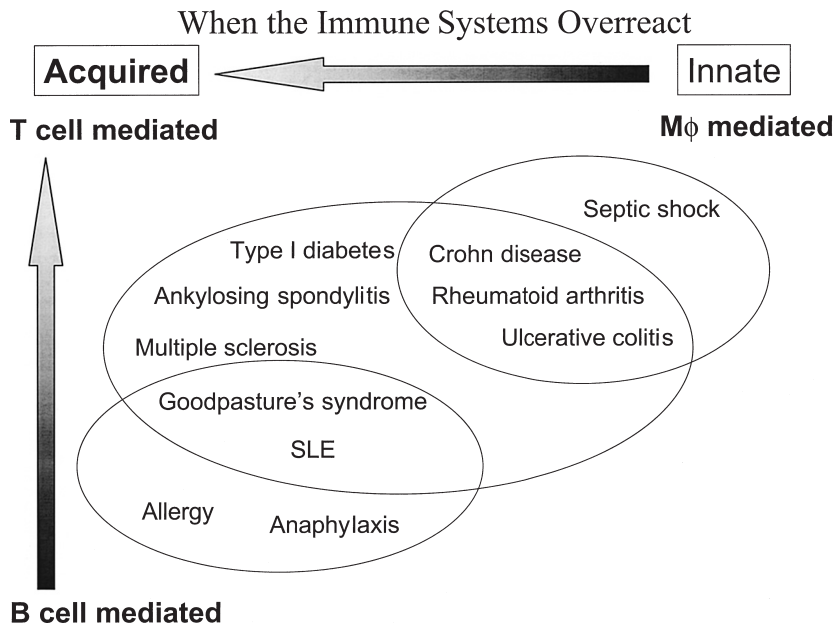


FIGURE 41.4. The spectrum of autoimmune diseases. Both innate and acquired immune dysfunction can cause disease in humans. While some autoimmune diseases involve principally cells of the acquired immune system, and at that, may be initiated by errors in B-cell or T-cell function, the innate immune system is frequently involved in pathogenesis as well, if only because of the close interplay that has evolved among cellular components of the immune system. Endotoxic shock may be taken as an example of autoimmunity in which the innate immune system alone plays a prominent role. *SLE*, systemic lupus erythematosus.

exponent of innate immune sensing may place us in a position to understand such diseases.

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