How we detect microbes and respond to them: the Toll-like receptors and their transducers

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Abstract: Macrophages and dendritic cells are in the front line of host defense. When they sense host invasion, they produce cytokines that alert other innate immune cells and also abet the development of an adaptive immune response. Although lipolysaccharide (LPS), peptidoglycan, unmethylated DNA, and other microbial products were long known to be the primary targets of innate immune recognition, there was puzzlement as to how each molecule triggered a response. It is now known that the Toll-like receptors (TLRs) are the principal signaling molecules through which mammals sense infection. Each TLR recognizes a restricted subset of molecules produced by microbes, and in some circumstances, only a single type of molecule is sensed (e.g., only LPS is sensed by TLR4). TLRs direct the activation of immune cells near to and far from the site of infection, mobilizing the comparatively vast immune resources of the host to confine and defeat an invasive organism before it has become widespread. The biochemical details of TLR signaling have been analyzed through forward and reverse genetic methods, and full elucidation of the molecular interactions that transpire within the first minutes following contact between host and pathogen will soon be at hand. *J. Leukoc. Biol.* 74: 479–485; 2003.

Key Words: *lipopolysaccharide · MD-2 molecule · interleukin-1 · TNF · infection · sepsis · adjuvant · interferon · innate immunity*

INTRODUCTION

Living organisms seem more than the sum of their parts, and this very paradox might be taken to suggest that there are limits to what we can know about them. The new school of "systems biology" rests on the premise that complex phenomena can best be understood by observing many events at once. How else can one hope to understand consciousness, development, or immunity? In each example, many separate events contribute to the whole phenomenon and do so simultaneously and in many instances, synergistically.

At the same time, vast progress in biology has come from reductionism. The search for the primary cause of events has often led investigators to disrupt individual molecules. This, in turn, has allowed inferences about precisely what is required for a biological system to function as it does. In time, these inferences may accumulate so that every requirement for proper function of a complex system is understood, much as every enzyme in the tricarboxylic acid cycle is now known. In no field has reductionism played a greater role than it has in the science of genetics. Mutations have fueled progress in genetics, and genetic tools have transformed every area of biology.

The present review concerns innate immunity: the inherited resistance to infection, shared to some extent by all normal metazoans. We would like to understand innate immunity at a fine molecular level. We would like to catalog each of the biochemical events that innate immunity entails so that every protein participant in the innate immune response is known. We would like to understand the temporal sequence of molecular interactions that occur and the importance of this temporal sequence to the outcome that is observed. In the long run, we might wish to modify innate immune responses, damping them when they cause harm (as they do in the course of inflammatory diseases) and augmenting them when they are required (as they are during focal infections that need to be checked before they become generalized).

INFLAMMATION AND SEPSIS AS BIOCHEMICAL PHENOMENA AND THE RELATIONSHIP BETWEEN THEM

Innate immunity and inflammation are not synonymous terms, but inflammation arose primarily to deal with infection. Therefore, to understand precisely how inflammation is initiated under any conditions and to trace the activation events that occur within cells of the innate immune system, it is necessary to identify individual molecules of microbial origin that act as inducers of inflammation and the host receptor molecules that detect them. Fortunately, a large number of microbial inducers have been identified. The earliest attempts to isolate these inducers and solve their structures were driven by the most basic question in microbial pathogenesis: How do microbes cause disease?

Undoubtedly, the most celebrated of these was lipopolysaccharide (LPS), the principal glycolipid component of the outer membrane of Gram-negative bacteria. The primary structure of

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LPS was solved during the early 1970s [1]. In addition, cord factor (trehalose dimycolate) [2], peptidoglycan [3], double-stranded RNA [4], and unmethylated DNA [5] were all shown to induce innate immune responses and to have adjuvant effects as well (see ref. [6] for a review).

As LPS could reproduce many of the features of an authentic Gram-negative infection and as it was easy to produce and store, it was widely used as an inducer of immune responses even in the absence of information concerning its receptor. The nature of the LPS sensor, presently believed to have three essential subunits, became clear in three stages. First, CD14 was shown to be essential for LPS responses [7]. Second, positional cloning revealed the membrane-spanning component of the receptor [Toll-like receptor 4 (TLR4); refs. 8, 9]. Finally, a small, exteriorized molecule known as MD-2 was shown to be an essential part of the receptor complex [10, 11]. The existence of still other components cannot, at this point, be excluded [12].

THE TLR FAMILY AND ITS LIGANDS

When TLR4 was first identified as the membrane-spanning component of the LPS receptor complex, four similar proteins were known to exist in mammals, each marked by similarity to the *Drosophila* protein Toll. In no instance was the function of these receptors known, although it was alternately postulated that developmental [13] or immunologic [14] roles might be played by the TLRs. The first TLR (now known as TLR1) was identified in 1994 [13, 14], TLR4 was identified in expressed sequence tag (EST) libraries in 1997 [15], and TLR2, -3, and -5 were cloned shortly thereafter, also on the basis of EST homologies [16–18], as was TLR6 also [19]. With the completion of the human genome sequence [20], a total of 10 TLRs were identified, and their cDNAs were cloned [21–23]. Eleven TLRs are known to exist in the mouse; these include the orthologs of human TLR1 through -9 and two additional TLRs, mapping to chromosomes 4 and 14. The latter are known to be expressed as their products are detected in EST libraries (B. Beutler, unpublished observation). However, the mouse TLR10 ortholog has been mostly deleted, and only a small fragment remains in the genome.

The signaling domain of each of the TLRs [the so-called TIR domain, denoting TLRs, interleukin-1 receptors (IL-1Rs), and plant disease-resistance genes] had some years earlier been recognized as an immunologically important motif. In *Drosophila*, Toll was shown to be required for defense against fungal infection [24]; in mammals, IL-1 and IL-18 were known to be involved in innate and adaptive immune responses [25]. IL-1 [26] and Toll [27–29] were known to signal through activation of nuclear factor-κB (NF-κB), and subsequently, soon after its identification, it was shown that TLR4 could do so as well [15].

Toll of *Drosophila* does not directly engage microbial ligands but rather a protein ligand (Spaetzle) [30]. However, the mammalian TLRs apparently do. Genetic [31, 32] and biochemical [33] evidence of interaction between LPS and TLR4 has been presented. LPS is first engaged by CD14 and then brought into direct contact with TLR4 and MD-2 [11, 34]. By implication, other TLRs may also bind microbial inducer molecules, as discussed below. Indeed, the case has been established for TLR9 with regard to DNA binding [35] and for TLR2 with regard to peptidoglycan binding [36] using methods similar to those used for TLR4. However, the TLRs may require accessory proteins for ligand presentation, and some genetic evidence suggests that this is the case.

The discovery that TLR4 signals the presence of LPS—and the strong likelihood that it exists exclusively for this purpose—made it seem probable that the other TLRs could each signal the presence of other microbial inducers. In this manner, much of the microbial world might be recognized by a handful of innate immune receptors. A combination of reverse genetic methods was used to demonstrate that this was the case. Underhill et al. [37] applied a dominant-negative approach to implicate TLR2 as the receptor for molecules of Gram-positive origin, an impression substantiated by the discovery that knockout of TLR2 made mouse macrophages refractory to activation by peptidoglycan and bacterial lipopeptides [38]. Later, TLR1 and -6 were shown to engage in heteromer formation with TLR2 [39], each contributing to a different specificity of recognition [40]. TLR5 was shown to recognize flagellin [41], a conclusion based on the effects of mutations in bacteria rather than mutations in the host (i.e., bacterial strains that lack flagellin fail to induce a response in cells transfected to express TLR5). TLR9 and -3 were shown to identify unmethylated DNA and double-stranded RNA, respectively [42, 43]. Finally, although an authentic microbial product has not yet been identified for TLR7, small, antiviral molecules with a nucleoside structure (imiquimod and resiquimod) were shown to stimulate cells via TLR7 [44]. In humans, it is believed that TLR8 may also act as a receptor for these ligands [45].

Although a large number of other molecules have been offered as putative TLR ligands as well, a conservative view of the specificities is presented in **Figure 1**. In the case of TLR4 itself, many other agonists have been proposed, including endogenous and virally encoded proteins. However, most have not yet been substantiated in systems that would entirely exclude the possible action of LPS, and at present, only LPS and taxol (the latter being an agonist for mouse TLR4 but not human TLR4) are accepted as proven ligands by the authors of this review.

WHAT THE TLRs DO AND WHY THEY ARE SO IMPORTANT

The germ theory of disease, first propounded by Pasteur and Koch more than 100 years ago, was a landmark advance, as it ascribed all of the consequences of an infection—no matter how complex they might be—to microbes. It remained for microbiologists to determine precisely which microbial molecules were of key importance to the causation of injury. LPS, discovered early on by Pfeiffer [46], was one such molecule and peptidoglycan another; still others emerged in turn. The avenue by which each of these molecules worked remained unclear until very recently.

The discovery of the TLRs as primary sensors of microbial infection was also a landmark advance, as the TLRs are the most proximal host initiators of septic shock and for that

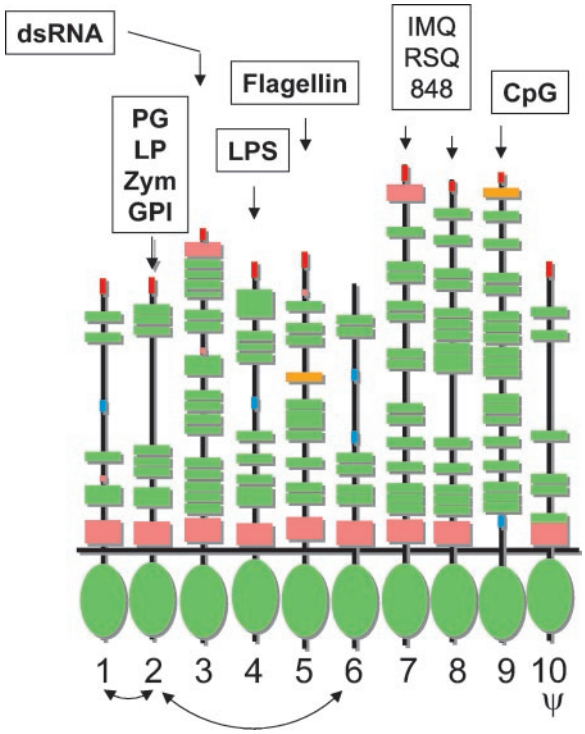

Fig. 1. The human TLRs and their specific ligands. Leucine-rich repeat (LRR) motifs are scattered throughout the ectodomains and are depicted as green rectangles; membrane-proximal LRRs have a different structure and are shown in pink. Green ovals represent TIR domains, the most conserved part of each of the receptors. TLR10 is a pseudogene in mice, which have two additional TLRs (not illustrated here) that are pseudogenes in humans. TLR1 and -2 and TLR-6 and -2 are known to form heterodimers. PG, peptidoglycan; LP, bacterial lipopeptides; zym, zymosan; GPI, glycosylphosphoinositol; IMQ, imiquimod; RSQ, resiquimod; 848, another congener of imiquimod and resiquimod; CpG, unmethyated DNA with immunostimulatory CpG dinucleotide-containing sequences.

matter, most of the responses that infectious organisms provoke. The TLRs stand at the top of the innate immune response cascade, crossing the membrane of host responder cells. They are the most important interface between mammalian host and microbe. Without them, there would be very little in the way of awareness that infection had occurred.

Just as they precipitate the "bad" effects of infection, it is clear that the TLRs protect against infection. Since the 1980s, the *Lps* mutation (now known to reside within the TLR4 gene) has been known to cause impaired responses to Gram-negative bacterial infection by entirely preventing host recognition of LPS [47]. Hence, Gram-negative bacteria may overwhelm the host by stealth. A small, Gram-negative inoculum, normally containable, may gain a foothold in an animal "blind" to endotoxin.

It is difficult to catalog the end effects of TLR activation, as these effects are so numerous, and one comes to a blanket description of inflammation itself. The intended effects (untoward if excessive or generalized) include recruitment of leukocytes, activation of microbicidal activity (i.e., the production of oxygen radicals, antimicrobial peptides, and hydrolytic enzymes), the relaxation of blood vessels mediated by nitric oxide (NO) and by autacoids, and abetment of the adaptive immune response (i.e., an adjuvant effect). Conversely, as a distinct biochemical cascade, which is initiated by receptor activation itself, elicited each of these end processes, much attention has been devoted to the proximal events that follow TLR stimulation.

SIGNALING FROM THE TLRs: THE BIOCHEMICAL DETAILS

Although not all components of the TLR complexes have necessarily been found, it may be inferred that ligand association with TLR ectodomains yields a conformational change that is sensed in the cytoplasmic compartment. For all of the TLRs, recruitment of MyD88—a cytoplasmic protein with a TIR domain that serves as an adaptor—is a crucial event, and many (but not all) TLR signals are abolished by targeted deletion of the MyD88 gene [42, 48–51]. Indeed, knockout studies defined "MyD88-dependent" and "MyD88-independent" pathways of responses to LPS, which signals via TLR4 [48].

The MyD88-dependent pathway entails recruitment of IL-1R-associated kinase (IRAK) isoforms (IRAK4 being particularly important [52]), tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF-6) [53, 54], and transforming growth factor-β-activated kinase-1 (TAK-1) [55] and activation of the signalosome, with subsequent translocation of NF-κB to the nucleus and the transcriptional activation of numerous cytokine genes.

The MyD88-independent pathway leads to the activation of the interferon-β (IFN-β) gene [48, 56], and presumably other genes dependent on IFN regulatory factor-3 (IRF-3) activation. Type I IFNs, acting through their own receptors, activate the Janus kinases, which in turn, phosphorylate signal transducer and activator of transcription (STAT) proteins, leading to the transcriptional activation of other genes, including many that are required for effective antiviral defense and other genes encoding proteins involved in the inflammatory response, such as the IFN-inducible protein 10 (IP-10) gene and the inducible NO synthase (iNOS) gene [56].

In the case of TLR4, association with at least three TIR domain-containing proteins occurs. The first of these, discussed above, is MyD88. However, in addition, MyD88 adapter-like [MAL; also known as Toll/IL-1R domain-containing adapter protein (Tirap); refs. 57, 58] and TIR domain-containing adapter-inducing IFN-β (Trif) [59, 60] recruitment also takes place. Targeted deletion of the MAL/Tirap gene yields a phenotype similar to that observed with MyD88 deletion, affecting not only the TLR4 receptor complex but also the TLR2 receptor [61, 62]. Trif mutants have not yet been generated, but it is likely that the Trif protein serves a MyD88-independent pathway of response (**Fig. 2**). Trif binds to numerous TLRs, as well as to IRF-3, and dominant-negative mutants of Trif seem to block signaling via IRF-3, preventing the production of IFN-β [59].

It is somewhat surprising that stimulation of the LPS receptor can elicit an antiviral state, although viruses themselves have not been convincingly shown to cause activation of the LPS receptor. IRF-3 itself, a transcription factor, undergoes phosphorylation and dimerization during viral infection of the

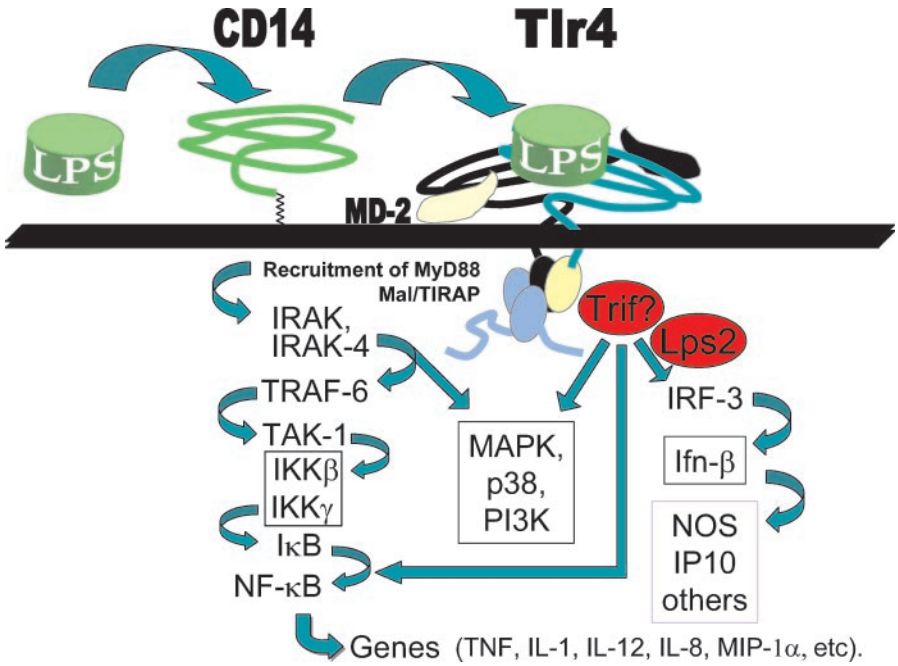

Fig. 2. The principal pathways of LPS signal transduction. MyD88-dependent signaling (left) depends on MyD88 and MAL/Tirap. MyD88-independent signaling (right) is clearly known from forward genetic studies to be dependent on the product of a gene known as *Lps2* and on the basis of transfection studies, may also depend on Trif, one of five adaptor proteins known to be present in the human and mouse genomes. Both pathways may activate NF-κB and the mitogen-activated protein kinase (MAPK) cascade. Only the MyD88-independent pathway activates IFN-β production. IKK, IκB kinase; PI-3K, phosphatidylinositol-3 kinase; MIP-1α, macrophage-inflammatory protein-1α.

cell. However, the proximal cause of this event (i.e., the kinase involved) has never been identified. It now appears that at least TLR3 (which detects double-stranded RNA) and TLR4 (which detects LPS) can cause IRF-3 activation [63]. Hence, at least two of the TLRs activate the MyD88-independent pathway. However, there are clearly gaps that must be filled. At least five TIR domain-adaptor proteins exist in the mammalian genome and can be found by homology searches within publicly accessible EST databases.

THE FORWARD AND REVERSE GENETIC METHODS

The "reverse genetic" approach is one that holds that the function of any protein can be deciphered if the gene encoding that protein is intentionally modified, knocked out, or overexpressed. Moreover, the function of many proteins can be deduced from analysis of structure alone.

The "forward genetic" approach holds that all genes required for a particular biological function can be identified by finding mutations that disrupt the function in question. The forward genetic approach is driven by phenotype. Rather than beginning with a protein, one begins with functional alteration and then searches for a mutation that explains this alteration.

Forward genetic approaches (e.g., the identification of *Lps* as TLR4) and reverse genetic approaches (e.g., the knockout of the TLRs and TIR domain proteins accomplished to date) have been used to decipher the mechanisms of innate immune sensing. Each method opens the door to other approaches. For example, a systematic search for TIR domain proteins led to the identification of Trif and MAL/Tirap, and biochemical assessments of molecular interactions have disclosed the participation of other proteins, such as MD-2, in signal transduction.

As a generalization, forward genetic methods can yield real surprises and as it makes no prejudgments about function, can open entirely new fields for study. Molecules with no known function or molecules with functions that were not thought to be related to a particular phenomenon at all can be shown to be essential for that phenomenon to occur. A shortage of phenotypes limits the practice of forward genetic analysis. Where interstrain phenotypic differences exist, they often have a polygenic basis and are therefore difficult to clone. Monogenic phenotypes are therefore the most interesting and important to follow. To create monogenic phenotype, germline mutagenesis is often used, and the mutagen of choice is N-ethyl-N-nitrosourea.

Several germline mutations that impair innate immune sensing have been identified during the past few months. These include *Lps2*, a codominant mutation that prevents LPS [12] and poly I:C (K. Hoebe et al., submitted) sensing; *Panr1*, a mutation that blocks all signaling initiated by microbial inducers (K. Hoebe et al., unpublished); and *Pgn1*, a mutation that selectively impedes responses to peptidoglycan (but has no effect on signaling by other TLR2 agonists; K. Hoebe et al., submitted). The functional position of each of these mutations is shown in **Figure 3**.

Even without knowing what genes these mutations affect, it is possible to draw important inferences. As they do not involve loci encoding "core" components of the TLR signaling pathways, we may be certain that there are other proteins that we have not yet identified. As a single mutation can disrupt the sensing of one TLR2 agonist without an effect on others, we can deduce that there are specific coreceptors for the broad spectrum of ligands that TLR2 detects.

HOW DO THE TLRs USE DIFFERENT TRANSDUCERS, AND WHAT ARE THE IMPLICATIONS OF THIS

It has been shown [64] that LPS induces most of the transcriptional responses in dendritic cells (DCs), which are induced by

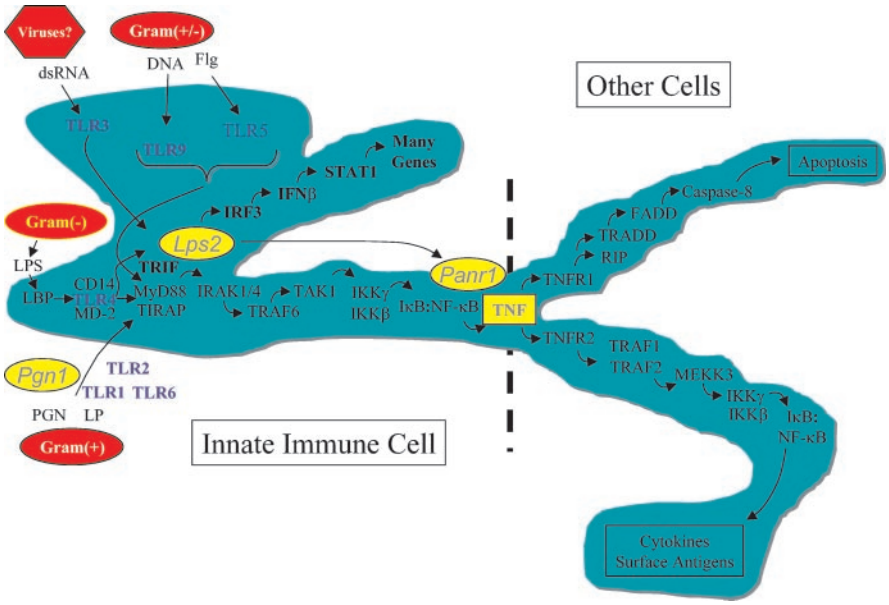

Fig. 3. Illustration of the principal signaling pathways activated by TLRs. Gram-negative and Gram-positive bacteria and perhaps viruses (red shapes) produce molecules that activate different TLRs or TLR complexes (blue), which in turn, impinge on a collection of transducer molecules. It is clear that TLR4, the LPS receptor, requires MyD88, MAL/Tirap, and other transducer molecules yet to be assigned. TLR2 requires only MyD88 and MAL/Tirap for signaling, so far as is known. TLR3 is perhaps somewhat dependent on MyD88 but also on other transducers. The integration of signals leads, in the case of the LPS response, to activation of the MyD88-dependent and MyD88-independent signaling pathways. TLR3 perhaps activates the MyD88-independent pathway exclusively. This pathway includes, among its many endpoints, the induction of IFN-β synthesis and all downstream events that follow STAT activation. Flux through the pathway is also required for effective TNF production. Among the principal endpoints of the MyD88-dependent pathway is NF-κB activation, on which TNF production also depends. TNF, a hallmark of LPS activation and one of the principal mediators of LPS toxicity, initiates two separate signaling pathways of its own (right). Forward genetic methods have been used to detect essential components of this pathway (yellow ovals). LBP, LPS-binding protein; PGN, peptidoglycan; LP, lipoprotein; FADD, Fas-associated death domain; TRADD, TNFR-associated death domain; RIP, receptor-interacting protein; MEKK, MAPK kinase kinase. Flg, flagellin. PanRI, "Pan-resistance I," a mutation known to block TNF production in response to all stimuli.

intact *Escherichia coli* organisms. As *E. coli* are capable of stimulating many TLRs, whereas pure LPS is capable of stimulating only TLR4, it can be concluded that many of the responses of a particular TLR are shared by all TLRs. Conversely, some responses to TLR4 are not elicited by TLR2 agonists [56], and in time, a catalog of specific responses might be assembled so that a given endpoint of response will have genuine diagnostic value.

Why is there so much overlap, and what accounts for the specificity that can be detected? In part, there is commonality of signal transducers. MAL/Tirap apparently serves TLR2 and TLR4 but not other TLRs. The specificity of Trif is not yet known with certainty: It has been claimed to serve TLR3 alone [60] or alternatively, several of the TLRs [59]. MyD88 is believed to be universal (i.e., nonspecific) and is required for signaling from the IL-1 and IL-18 receptors as well as the TLRs [65]. From germline mutagenesis studies, it appears that the protein encoded by *Lps2* serves only TLR3 and -4 [12].

There are 10 human TLRs and only three known transducers. It has also been shown that at least in some cases, TLRs engage in heteromer formation, broadening the specificity of molecules that are recognized. For example, TLR2 can combine with TLR1 or TLR6 and is likely to also exist as a homodimer [39]. It might therefore be expected that the informational potential of the receptors is lost in the course of signaling, as once a transducer is activated, the cell can no longer "know" which receptor was responsible. However, the ratio of activation of different transducers, the temporal relationship between activation of different transducers, and the subcellular location of the receptors and transducers might be influential in directing the output of the response. Therefore, although TLR2 and TLR4 might recruit MyD88, MAL/Tirap, and Trif, the ligands for these receptors do not yield identical responses. Furthermore, although TLR7, -8, and -9 are believed to reside within the cytoplasm of responding cells, perhaps anchored within the endosomes, TLR4 is at least partly located at the cell surface.

Beyond this, it is entirely possible that many of the proteins that serve the TLRs have yet to be identified. Some may indeed be entirely unique to particular receptors.

THE BRIDGE FROM INNATE TO ADAPTIVE IMMUNITY

Before the LPS-sensing role of TLR4 was known, it was proposed that this protein could "activate adaptive immunity" on the basis of the fact that TLR4 ligation could activate NF-κB [14]. It has been widely suggested that the TLRs are important in adaptive immunity, as they are in innate immunity. In fact, this is the case, although adaptive immune responses occur far downstream from TLR activation.

Although it is sometimes presented as a new concept, the dependence of adaptive immunity on cells of the innate immune system has been known for a very long time. The vital antigen-presenting role of innate immune cells accounts for much of this dependency. The assistance rendered by specific host molecules such as IL-1, CD40L, class II major histocompatibility complex antigens, and B7 antigens, each of which is produced or up-regulated in response to LPS or other microbial stimuli, was established decades ago. The adjuvant effect of molecules of microbial origin was evident earlier still (for a review, see ref. [6]).

An attractive hypothesis concerning the role played by TLRs in the activation of adaptive immunity holds that the precise combination of TLRs activated by a given microbial infection leads to "tailoring" the adaptive immune response so that it can deal with that specific infection. Hence, a DC, stimulated by

LPS, might direct the development of an adaptive response that is better suited for dealing with Gram-negative organisms than Gram-positive organisms. However, if true, this notion would be very difficult to prove, given the formal similarities of the adaptive response to all organisms.

Certain corollaries to the hypothesis that TLRs are critically important to the development of adaptive immune responses, although often cited, are difficult to sustain in the face of existing information about the immune response. One of these corollaries is the notion that self-tolerance prevails because of a lack of TLR signaling and hence, the lack of a costimulus in the form of CD80 [66]. However, the administration of a TLR agonist (or the introduction of an infectious agent) does not, by itself, break tolerance to the normal tissues of the host nor to tissues that have been damaged or destroyed as a result of the infectious process.

A second corollary holds that TLR activation provides an essential "second signal" for an adaptive immune response [67]. Although as adjuvants of all kinds enhance the adaptive immune response by definition, they are not essential for its occurrence. Immune responses to allografts, for example, can be exceptionally strong and occur in the absence of any TLR agonist. Immune responses to many viruses undoubtedly also circumvent the TLR-sensing system. Isolated foreign proteins are, of course, immunogenic as well, whether they are introduced as highly purified preparations in the absence of adjuvants or are made by recombinant means within the host (e.g., when encoded by a viral vector).

THE LIMITS OF INNATE IMMUNITY

Innate immunity has been refined over more than 1 billion years of evolution, and a rather large fraction of the host genome is devoted to defense. Still, more of the genome has dual functions and is devoted to defense on some occasions but not others. For example, some alleles of the human β-globin gene can protect the host from malaria, provided that the seventh codon specifies a valine (i.e., the sickle hemoglobin mutation). Is β-globin an innate immune protein? Perhaps innate immunity is in the eye of the beholder.

The element of timing is crucial to innate immune responses and to the determination of whether harm or good will come of them. Innate immune responses are not elicited all at once but in an optimal sequence. Such is the complexity of innate immunity that success in understanding it will hinge on the integration of systems biology and reductionism with all the tools that each can apply.

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