Commentary

Toll, A New Piece in the Puzzle of Innate Immunity

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Innate recognition of bacterial products constitutes a principal bulwark of our defenses against infection. In vertebrates, innate mechanisms both instruct the adaptive immune response and provide immediate protection from infectious challenge. In all lower phyla, innate mechanisms represent the totality of immune protection, attesting to both the power and evolutionary precedence of these mechanisms.

Although the workings of the adaptive immune system may be traced in a satisfying way from generation of diversity to ligation of surface immunoglobulin to clonal expansion, a similarly complete and satisfying view of innate recognition is not in hand. Even the best characterized example of innate recognition, the inflammatory response to bacterial LPS (endotoxin), has a yawning gap in the steps of its progression. Results of long anticipated work published in a recent issue of *Science* (1) and in this issue of *The Journal of Experimental Medicine* (2) have now identified a molecule with a privileged position in innate recognition and the potential to fill this major gap.

The Missing Pieces in the Innate Immunity Puzzle. Work on innate recognition of LPS has generated information from two polar starting points. The first has followed LPS and its binding partners during the initial interaction with cells. This work has shown that LPS is first acted on by LPS binding protein (LBP), a plasma lipid transfer protein that moves LPS monomers from aggregates or bacterial membranes to a binding site on CD14 (3, 4). CD14, a protein expressed both in plasma and as a glycosylphosphatidylinositol (GPI)-linked protein on the surface of leukocytes, then transfers LPS monomers into the plasma membrane of cells (references 3 and 5, and Vasselon, T., E. Hailman, R. Thieringer, and P.A. Detmers, manuscript submitted for publication). LPS moves in turn to the Golgi apparatus via an as yet undefined vesicular pathway (reference 6 and Thieblemont, N., and S.D. Wright, manuscript submitted for publication). The goal of this approach has been to identify the binding partner or "receptor" that discriminates LPS from host lipids and transduces signals across the membrane—but this goal has remained elusive: neither LBP nor CD14 has the binding specificity to discriminate LPS from host lipids (3, 7), and experiments to identify new binding partners for LPS in cells have been uniformly frustrating.

The second line of work began with gene expression in the nucleus and has worked backward toward determining the agents that initiate new transcription. LPS causes dramatic transcriptional regulation of a wide range of proinflammatory genes including TNF, IL-1, IL-8, IL-6, ICAM-1, E-selectin, tissue factor, and many more. LPS-dependent activation of these genes has been shown to be controlled by the transcription factors, nuclear factor (NF)-κB and AP-1. These transcription factors are in turn controlled by kinases that are rapidly activated by LPS (8, 9). For this approach as well, the still unmet goal is to identify the receptor that discriminates LPS from host lipids at the membrane and initiates the kinase cascade. The new work reviewed here focuses on the potential interface between the molecules defined by these two lines of investigation, and this article will focus on the extent to which a connection can be made.

Toll Proteins and the Response to LPS. Both recent studies discussed here (1, 2) examine the molecular defect in well-characterized mouse strains (C3H/HeJ and C57BL10/ScCr) that exhibit impaired ability to respond to LPS. Work on these mice over the past 20 yr has shown that hyporesponsiveness to LPS maps to a single autosomal locus (*lps*), and impaired responses can be documented both in whole animals and in cells taken from the animals (10). The consequence of this hyporesponsiveness is a dramatically enhanced susceptibility of lps^d animals to challenge with Gram-negative pathogens. Importantly, the animals respond normally to Gram-positive challenge and do not have other health defects. This phenotype corresponds precisely with that expected of a defect in innate recognition of LPS. It is fair to say that studies of lps^d animals did not merely "confirm expectations" about innate immunity. Rather, they showed for the first time that a functional innate mechanism for detecting particular microorganisms actually exists in mammals, that the range of microorganisms detected by this system is roughly defined as "Gram-negative bacteria," and that this mechanism plays an important role in resistance to infection. These studies have propelled research on LPS recognition as a paradigm of innate immunity. The identification of the genetic defect in lps^d mice has long been a holy grail, with the potential to reveal a protein at the heart of innate immunity, and that protein is now in hand.

Both Poltorak et al. (1) and Qureshi et al. (2) performed extensive genetic mapping to narrow the position of the *lps* locus to a region of chromosome 4, both assembled the target region on YACs and BACs, and both sought candidate genes on the basis of sequence and expression. However, for both groups, the most important clue came not from mapping but from studies in *Drosophila* which identified the Toll protein as a key player in the response to fungal infection (11). Several mammalian homologues of Toll have been discovered (see below), and recent studies had shown that TLR4 (Toll-like receptor 4, also known as hToll) can

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initiate signaling steps similar to those seen in response to LPS (12). Both Poltorak et al. and Qureshi et al. found TLR4 in the target region of chromosome 4, and both groups identified a missense mutation in the cytoplasmic domain of TLR4 in C3H/HeJ. Importantly, an independent mutation at the *lps* locus in the C57BL10/ScCr strain resulted in the absence of TLR4 message, offering a strong confirmation of the correct identification of TLR4 as the defective protein resulting in the lps^d phenotype. The formal proof that TLR4 accounts for impaired responses to LPS still must come from reconstitution of the defect with authentic sequence, but the data in hand provide fair assurance that the identification is correct.

The Toll Family and Signal Transduction. Toll is a transmembrane protein that was discovered as a necessary player in the establishment of dorsal-ventral polarity in *Drosophila* embryos (13). The cytoplasmic domain of Toll bears significant homology with the cytoplasmic domain of the IL-1 receptor, and Toll signals transcriptional changes in *Drosophila* through a cascade of proteins with remarkable similarity to those used by the IL-1 receptor:

IL-1R → MyD88 → IRAK → IκB → NF-κB → gene expression
Toll → Tube → Pelle → Cactus → Dorsal → gene expression.

In addition to its role in embryogenesis, Toll also plays an important role in antifungal defenses of flies, being necessary for the strong upregulation of drosomycin in response to fungal infection (11).

Recent observations from Medzhitov et al. (12) indicate that a human homologue of Toll (TLR4) can be an effective signaling molecule in mammalian cells. A constitutively active TLR4 construct drives NF-κB activation, AP-1 activation, and cytokine production in transfected cells (12). Additional data show that, like the IL-1R, TLR4 uses MyD88 and IRAK to activate NF-κ B (14, 15). As described above, NF-κB and AP-1 activation are key players in responses to LPS, and these data suggest that activation of a receptor such as TLR4 may be sufficient to explain the transcriptional responses to LPS. Together with the observation that the lps^d phenotype derives from defective TLR4, we may conclude that at least under some conditions TLR4 is necessary and sufficient for responses to LPS. These findings now extend our understanding of the steps in LPS signal transduction from the nucleus all the way to the membrane.

Additional data suggest that, as expected with any important physiological function, redundancy exists with respect to LPS signaling. Although lps^d mice are hyporesponsive to LPS, they are not unresponsive, and LPS-dependent gene transcription will occur if a very large dose of LPS is administered (10). Moreover, the sensitivity of lps^d cells and animals may be restored by activation with IFN-γ (16, 17), and cells from C3H/HeJ mice are nearly as sensitive as their normal counterparts when stimulated with certain types of LPS (e.g., *Porphyromonas gingivalis* LPS) (18). These observations suggest that proteins other than TLR4 may replace the function of TLR4 in signal transduction for responses to LPS.

The best candidates for backing up TLR4 are other members of the Toll family. In *Drosophila*, a homologue of Toll known as "18 wheeler" also plays a role in host defense but serves primarily in recognition of bacterial rather than fungal pathogens (19). In humans, five homologues of Toll have been described by three groups (12, 20, 21). Importantly, transfection of cell lines with TLR2 confers on them the ability to respond to LPS with activation of NF-κB (22, 23), thus directly suggesting that TLR2 may serve in place of TLR4. It is interesting to note that Kirschning et al. (23) examined not only TLR2 but also TLR4. Contrary to the prediction from the lps^d mice, transfection of TLR4 led to constitutive activation of NF-κB with no enhancement upon addition of LPS. The reasons for this discrepancy are not clear but could derive from the presence of the additional "Flag" sequence in the TLR4 construct or the requirement for a factor or subunit not present in the recipient cells. It is clear that a great deal of work needs to be done to fully describe the cell distribution, regulation, and contribution of individual members of the Toll family to responses to LPS and other microbial products.

Toll and Discrimination of LPS from Host Lipids. As outlined above, TLR4 appears to be an early and necessary part of the signal transduction machinery linking LPS with gene expression. Since TLR4 is a transmembrane protein, we may now ask the most important question: is TLR4 the critical "LPS receptor" that discriminates LPS from host lipids and initiates signaling? Is it the last piece in the puzzle, the "T cell receptor of endotoxin biology?" Use of Occam's razor makes this the obvious suggestion. On the other hand, any casual inspection of a diagram of signal transduction will reveal that nature has made scant use of Occam's razor in this area, and the remainder of this commentary will address the known and possible links between LPS and TLR4.

The extracellular domain of TLR4 contains 22 copies of a leucine rich repeat (LRR) motif. The best characterized binding partner of LPS, CD14, contains 10 copies of the LRR motif. Despite this marvelous similarity, it is unlikely that the LRRs of TLR4 represent an LPS binding site. Mapping studies with CD14 have revealed that 7 out of the 10 LRRs can be deleted without affecting LPS binding (24), and additional mapping studies have defined residues outside the LRR region of CD14 that are clearly necessary for LPS binding (25). Yang et al. (22) have suggested that LPS may bind directly to TLR2. However, the affinity and stoichiometry observed were so low as to eliminate any biological significance of this phenomenon, and at present there is no strong evidence for a direct, meaningful interaction of LPS with any member of the Toll family. An alternative to direct binding of LPS is an interaction of TLR4 with CD14. Although conceptually attractive, work with CD14 has failed to uncover a binding partner in cells. Despite use of probes of extremely high sensitivity, it has not been possible to measure binding of CD14 (26) or CD14-LPS complexes (Vasselon, T., and R. Thieringer, unpublished observations) to LPS-responsive cells.

A key to understanding how LPS might interact with TLR4 (or any other adapter protein) comes from a consideration of the form and location of the LPS during recogni-

tion. LPS is an amphiphile that will incorporate into lipid bilayers. Indeed, CD14 readily donates LPS to liposomes, lipoprotein particles, and the membranes of responsive cells (references 5, 27, and 28, and Vasselon, T., E. Hailman, R. Thieringer, and P.A. Detmers, manuscript submitted for publication). Two lines of evidence suggest that LPS signals responses not as a monomer but as a component of the lipid bilayer. Scientists at Eisai Pharmaceuticals have synthesized an LPS homologue, E5531, that acts as a powerful antagonist of LPS action in cells and animals (29). Inversion of all 13 chiral centers of E5531 yields a mirror image, and this mirror image compound shows equal ability to antagonize LPS action (Christ, W.J. 1998. Advances in synthetic LPS antagonists. Oral presentation at Fifth Conference of the International Endotoxin Society, Santa Fe, NM). This finding argues that LPS is not recognized in the stereospecific fashion expected of stoichiometric interactions with proteins. An alternative is that the colligative properties, which are identical in enantiomers, may be key to the biological action of E5531 and LPS. This possibility is suggested by the finding that another LPS analogue (from *Rhodobacter sphaeroides*) may be converted from an antagonist to an agonist by simultaneous addition of the membrane-active agent, chlorpromazine (30). From these considerations, we are directed to seek recognition proteins that either sense LPS in a bilayer or that sense the properties of a bilayer containing LPS.

There is only one well-characterized precedent in which membrane composition is sensed and relayed to gene expression. The concentration of cholesterol in membranes of the endoplasmic reticulum is gauged by a set of proteins that control transcription of the genes for cholesterol synthesis and uptake and which thereby affect cholesterol homeostasis at the cellular level (for review see reference 31). In brief, low levels of cholesterol in the endoplasmic reticulum are thought to be sensed by a multispan protein known as SCAP. SCAP controls the action of a transmembrane protease (S1P). Interestingly, the proteolytic domain of S1P is lumenally disposed (32), and its substrate is an intralumenal loop of SREBP (sterol response element binding protein), a transcription factor. Cleavage of the SREBP by S1P in turn enables a second cleavage on the cytoplasmic face of SREBP, and this cut liberates a fragment of SREBP that acts as a transcription factor. For the purposes of this discussion, the message I wish to take from this diversion is that membrane composition can be sensed and that, at least for cholesterol, the actuation device is a protease cascade that starts in the lumen of an intracellular vesicle. For completeness, it should be added that just as Toll is involved in dorsal-ventral polarity in *Drosophila*, other genes known as *hedgehog* and *patched* are involved in establishing anterior-posterior polarity (33). *Hedgehog* encodes a protein covalently derivatized with cholesterol, and *patched* encodes a homologue of SCAP.

Several observations suggest that LPS may activate a protease cascade and that a protease cascade may play a role in activation of receptors such as Toll. In *Drosophila*, the ligand for Toll that drives dorsal-ventral polarity is a proteolytic fragment of the protein, spätzle. Spätzle is cleaved by the protease, easter, and easter in turn is activated by another protease known as snake (13). It is also well known that LPS can initiate a protease cascade that leads to clotting in arthropods, and the familiar *Limulus* amebocyte lysate (LAL) assay for LPS exploits this cascade. Recent studies indicate a high similarity between the LPS-induced clotting cascade and the Toll cascade (34). Snake and easter are homologues of the *Limulus* proteases factor B and proclotting enzyme, respectively. The final step of the LAL reaction involves cleavage of coagulogen. As its name implies, coagulogen forms a fibrin-like clot but recent crystal structure analysis (35) has revealed that, in addition, coagulogen contains a cysteine knot fold also found in spätzle, NGF, TGF-β, and other signaling molecules. It is thus a reasonable possibility that LPS may initiate a protease cascade to generate ligands for receptors such as Toll.

Could a protease cascade function in recognition of LPS in mammalian cells? The only soluble factors needed for responses of mammalian cells to LPS in vitro are LBP and CD14, arguing against a role for a plasma-borne protease cascade. An alternative source of a cascade is the cell itself with the proteases being membrane bound. Many transmembrane proteases have been characterized and are known to function in sensing cholesterol concentration (described above), processing of *notch* (36), and maturation of signaling proteins such as insulin (37). Additionally, since LPS is rapidly internalized and carried to the Golgi, soluble components may exist in sufficient concentration in this organelle. It is worth noting that like LPS, ceramide is also transported rapidly to the Golgi (38) and *lps*^{d} cells fail to respond to ceramide (39). Membrane-bound proteases such as furin are known to be concentrated in the Golgi (37), certain genes that act upstream of Toll (pipe and windbeutlel) are found in the Golgi (13), and the IL-1R has been reported to traffic to the Golgi after ligation (40).

In conclusion, the new work describing a role for TLR4 in responses to LPS clearly adds a piece to the puzzle of LPS responsiveness and innate immunity. How important is this piece? TLR4 clearly contributes a new part of the signal transduction cascade but it is not clear that TLR4 contributes to the most vexing problem in innate immunity: how do cells discriminate LPS or other microbial molecules from similar structures in the environment? Work in the next year should clarify this issue, but this author's view is that TLR4 may be well downstream of the step or steps discriminating host from pathogen.

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