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*J Immunol* 2006; 177:11-16; doi: 10.4049/jimmunol.177.1.11
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Intracellular TLR Signaling: A Structural Perspective on Human Disease

Michael V. Lasker2*† and Satish K. Nair*‡

TLRs are crucial sensors of microbial infection. Maintaining structural integrity of TLR signaling components is essential for subsequent immunoprotective action. Alterations to the structure of these signaling molecules are often associated with profound clinical outcomes and susceptibility to various infectious diseases. These changes in structure are sometimes the result of a single nucleotide polymorphism (SNP). Numerous SNPs have been found in components of the TLR signaling pathway. Recently, the medical consequences and effects on TLR signaling of several of these SNPs have been elucidated. In addition, there are numerous structures solved that are important to our understanding of the TLR signaling pathway at the molecular level. The scope of this review is to tie together current structural, biochemical, and genetic information of TLR signaling. The Journal of Immunology, 2006, 177: 11–16.

The mammalian innate immune system sounds the first alarm that an infection is present. The response that it elicits happens within a matter of minutes and provides the first line of defense against infection. Innate immunity relies on the TLRs as the key sensors of microbial infection in mammals. As the main sensors of the innate immune system, TLRs modulate the induction of hundreds of host genes through a complex network of signaling that allows for an appropriate response to a given microbial pathogen. TLRs are comprised of an ectodomain with leucine-rich repeats, a transmembrane region, and a cytoplasmic Toll/IL-1R (TIR)3 domain so named for its homology to the IL-1R (Fig. 1). The TIR domain of the TLR interacts with a set of TIR domain-containing adaptor proteins (reviewed in Ref. 1). The universal adaptor protein, MyD88, serves to recruit a family of kinases known as the IL-1R-associated kinases (IRAKs) (2), eventually culminating in the activation of NF-κB and the production of proinflammatory cytokines (Fig. 1). The underlying molecular details of this signaling process are critical for the immunological protection of humans and other mammalian species.

Numerous examples of molecular perturbations to this signaling cascade have been identified, often with profound implications on human disease states. A single nucleotide polymorphism (SNP) discovered in TLR5 that results in the deletion of the entire signaling domain has been found to increase susceptibility to Legionnaires’ disease, but this same SNP is protective in the development of the autoimmune disorder, systemic lupus erythematosus (3, 4). Downstream signaling molecules of the TLRs such as IRAK-4 are also imperative for immunological protection. Humans with complete IRAK-4 deficiency or who have a truncated form that effectively deletes the kinase domain are inflicted with recurrent pyogenic bacterial infections early in life (5, 6).

In addition to these gross structural changes (i.e., domain deletions) found in human TLR signaling molecules, smaller perturbations can have profound effects on downstream signaling and the production of proinflammatory cytokines (7). For example, mice bearing the P712H mutation of Tlr4 are completely resistant to endotoxic shock. This proline residue of Tlr4 is conserved throughout many TIR domains in both TLRs and their TIR-domain containing adaptors. These functional studies and others underline the importance of maintaining structural integrity of the innate immune components of intracellular TLR signaling. The molecular structures for a number of proteins and protein domains in TLR signaling have been determined (Table 1). These structures have assisted us in understanding innate immunity at the molecular level. This review will describe recently determined structures involved in TLR signaling, as well as tie together current structural, biochemical, and genetic information of the TLR pathway.

TIR domain: the bridge between receptor and cytoplasm

The TIR domain, named for the homology between the cytoplasmic portion of the Toll and IL-1 receptors, is typically between 135 and 160 residues in length (8). Sequence conservation among TIR domains is generally between 20 and 30%, and most of the conservation resides within the hydrophobic core of the domain. The differing length and significant variation in sequences of the TIR domain suggest that considerable structural diversity might exist. This diversity has been proposed to

1 This work was supported by start-up funds and the Institute for Genomic Biology, at the University of Illinois at Urbana–Champaign (to S.K.N.). M.V.L. was supported by National Institutes of Health National Research Service Award 1 F30 NS 048779-01 from the National Institute of Neurological Disorders and Stroke.
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E-mail address: lasker@uiuc.edu
3 Abbreviations used in this paper: TIR, Toll/IL-1R; IRAK, IL-1R-associated kinase; SNP, single nucleotide polymorphism; DD, death domain; dMyD88, Drosophila MyD88; ID, intermediate domain; MyD88s, splice variant of MyD88.

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Received for publication March 8, 2006. Accepted for publication March 29, 2006.

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be important in ensuring the formation of the correct components of the signaling complex takes place among the TLRs and IL-1Rs (8). Despite this diversity, the TIR domain signaling complex is conserved from insects to mammals. The complex is composed of the cytoplasmic portion of the receptor and one of the five TIR domain-containing adaptors: MyD88, Trif/Ticam, Tirap/Mal, TRAM, or SARM. TLR2 and 4 are known to be able to associate with multiple adaptors, including MyD88 and Tirap/Mal. Homotypic protein-protein interactions are thought to take place between the receptor and the adaptor molecule in order for signal transduction to occur (8) (Fig. 1).

The structures of the human TLR1 and TLR2 TIR domains provided us the first opportunity to visualize this domain at a molecular level. Both overall structures revealed a five-stranded parallel β-sheet that is surrounded by five helices (8) (Fig. 2A). β-strands and α-helices alternate with structured loops of varying length connecting them. Between the second β-strand (β2) and the second α-helix (α2), referred to as the bb loop, there is a large surface patch, which by primary sequence analysis is conserved in many TLRs. The TLR2 P681H mutation, which has been shown to be homologous to the Tlr4 P712H mutation (9), resides in this bb loop. The structure for the TLR2 P681H was also solved (8), but surprisingly had little effect on the overall structure of the TIR domain or the bb loop peptide backbone. Therefore, Pro<sup>681</sup> (Fig. 2B) is not essential to the structural integrity of either the TIR domain or the bb loop. This finding led to the hypothesis that the addition of the histidine side chain, introduced by the P681H mutation, must cause a disruption of a direct point of contact with another molecule. This molecular interference would prevent downstream signaling from taking place. To test this hypothesis, Xu et al. (8) mutated the most conserved residues within the bb loop and tested their functional effects using transfection assays with human TLR4 and Drosophila Toll. The results demonstrated that changing any of these residues leads to a significant reduction in receptor-mediated NF-κB activity. In addition to these transfection studies, protein-binding studies with the TIR domains of MyD88 and TLR2 were performed. Classical GST pulldown

Table 1. Structures solved in the Toll, IL-1R, or TLR signaling pathways

<table>
<thead>
<tr>
<th>Name</th>
<th>Organism of Origin</th>
<th>Complex or Alone</th>
<th>Domain(s) Solved</th>
<th>Disease State/Biological Significance</th>
<th>Structural Point Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>Mouse</td>
<td>Alone</td>
<td>N/A</td>
<td>LPS binding</td>
<td>None</td>
</tr>
<tr>
<td>TLR3</td>
<td>Human</td>
<td>Alone</td>
<td>Ectodomain</td>
<td>dsRNA recognition</td>
<td>None</td>
</tr>
<tr>
<td>TLR1</td>
<td>Human</td>
<td>Alone</td>
<td>TIR</td>
<td>Bacterial cell wall component recognition</td>
<td>None</td>
</tr>
<tr>
<td>TLR2</td>
<td>Human</td>
<td>Alone</td>
<td>TIR</td>
<td>Bacterial cell wall component recognition</td>
<td>P681H C713S</td>
</tr>
<tr>
<td>IL-1RAPL</td>
<td>Human</td>
<td>Alone</td>
<td>TIR</td>
<td>Unknown</td>
<td>None</td>
</tr>
<tr>
<td>Tube/Pelle</td>
<td>Drosophila</td>
<td>Complex</td>
<td>DD</td>
<td>Antifungal response</td>
<td>None</td>
</tr>
<tr>
<td>Pelle</td>
<td>Drosophila</td>
<td>Alone</td>
<td>DD</td>
<td>Antifungal response</td>
<td>None</td>
</tr>
<tr>
<td>IRAK-4</td>
<td>Mouse</td>
<td>Alone</td>
<td>DD</td>
<td>Deficiency leads to recurrent bacterial infections</td>
<td>None</td>
</tr>
</tbody>
</table>
assays with in vitro-transcribed and -translated (using rabbit reticulocyte lysates) TLR2 and P681H constructs were conducted. Although protein binding was quite robust between MyD88-TIR and wild-type TLR2-TIR, almost no interaction was detectable with the P681H mutant (8). A SNP in humans located within the bb loop of TLR2, R677W, is strongly correlated with lepromatous leprosy (10). The positively charged Arg677 serves to tether the bb loop to the end of the first helix by forming an ion pair with the negatively charged and strictly conserved Glu663 in the TLR2 structure (8) (Fig. 2B). Unlike the P681H mutation, which maintains the structural integrity of the bb loop, the R677W mutation would unpin the bb loop from the first helix and perturb its structure. This disturbance of the bb loop structure explains the functional findings of Bochud et al. (11) and their characterization of the TLR2 R677W polymorphism. They found this SNP variant to be nonfunctional and unable to mediate signaling. Activation of NF-kB by this human TLR2 polymorphism was completely abolished in response to Mycobacterium leprae and Mycobacterium tuberculosis (11). Given the importance of maintaining the structural integrity of the side chains and backbone of the bb loop for proper downstream signaling to occur, it has been targeted for specific inhibition. In fact, the normal interaction between IL-1R and MyD88 TIR domains can successfully be inhibited by a low m.w. mimic of the three protruding residues in the bb loop (12). The MyD88 TIR domain has been known to homodimerize (13), and this self-association can also be inhibited by peptides that target the bb loop (14). While these inhibitors or their derivatives might serve as useful therapeutics in the treatment of arthritis and chronic inflammatory disorders (14), care must be taken to avoid infections because inhibitors of the bb loop likely have a broad range of specificity within the TLRs and their adaptors.

R753Q, another SNP that occurs in humans that is not located in the bb loop, is linked with a hyporesponsiveness to bacterial peptides derived from Borrelia burgdorferi and Treponema pallidum (15). Analysis of the TLR2 structure reveals that Arg753 makes polar contacts with the backbone and side chains of the dd loop (Fig. 2B). Replacement with glutamine reduces the number of possible polar contacts and would most likely disturb the structure of the dd loop. In addition to the importance of maintaining the structural integrity of the bb and dd loops, it has also been proposed that dimerization of the TLR2 TIR domain is vital for downstream signaling to occur (16). Although the inherent affinity for self-association of the TIR domains was found to be rather low with dissociation constants in the millimolar range (8), a dimer of the TIR domain was seen in the C713S mutant TLR2 structure (16). Point mutations of the residues located in the dimeric interface were found to abrogate NF-kB activation in response to peptidoglycan stimulation. Four new mutations located at the dimeric interface (outside of the bb loop): C713S, L717E, R748E, and R748S were found to severely hamper NF-kB activation, presumably due to a failure of TLR2 to dimerize (16).

A dimer was also observed in the human IL-1RAPL TIR domain structure (17). The function of this receptor is largely unknown, but this is the only structure of a TIR domain solved within the IL-1R superfamily to date. Primary sequence analysis revealed that this dimeric interface may be unique to this receptor as the residues located within this interface are not conserved in other TIR domains. Although a dimer was seen in the crystal structure, it is known to behave as a monomer even at high concentrations such as was seen in the wild-type TLR1 and 2 structures (17). The overall structure is very much similar to the TIR domain structures of TLR1 and 2 with the exception of the dimeric interface and some conformational differences in the loops and the first α-helix. This conformational variability within TIR domains of these receptors might be responsible for maintaining specificity in the signal transduction process. The surface of the TIR domain is obviously important in determining downstream molecular interactions that will take place. As there have been no crystal structures of TIR domains of any of the adaptor proteins or complex crystal structures of a TLR bound to an adaptor, these molecular determinants have yet to be elucidated. Despite this lack of structural information, molecular models of the TIR domain of Tirap/Mal and MyD88 have been produced (18). These molecular models suggest that the overall fold is similar, but have different electrostatic surface potentials. According to docking studies performed with the two adaptors and TLR4, Tirap/Mal and MyD88 bind to two distinctly different regions of the receptor (18). An extensive mutational analysis of the TLR4 TIR domain also revealed at least two important surface patches for signaling (19).

The TIR domain interactions between receptor and adaptor are presumably crucial for TLR signaling to occur. In fact, a vaccinia virus immune evasion strategy using a TIR domain-containing viral protein, A46R, targets MyD88, Tirap/Mal, Trif/Ticam, and TRAM and interferes with the downstream activation of MAP kinases and NF-κB (20). In this study, A46R was shown to directly associate with MyD88 and inhibit MyD88 dependent signaling by both IL-1R and TLRs, presumably by interacting via its TIR domain. Interestingly, the A46R protein was also able to immunoprecipitated with TLR4 itself suggesting that the TIR domain of TLR4 might also be a target. This viral protein also antagonizes Trif/Ticam-dependent pathways and is expressed in the early stages of infection. Knockout of the A46R gene attenuates the virus highlighting its crucial role in virulence. Variola virus, the causative agent of smallpox, also possesses this TIR domain-containing molecule and likely uses similar mechanisms to evade the host’s immune system (20).

Mining Drosophila for structural details of the innate immune pathway

Researchers in the field of innate immunity have certainly gained insight from the model organism Drosophila melanogaster, beginning with the initial discovery that the Toll receptor played an important immunological role in combating fungal infections in flies (21). It was only after this discovery when researchers began to find that human homologs of Toll (i.e., TLRs) had an immune function. Structural biology is no exception; Drosophila proteins have helped us understand Toll signaling at the molecular level. Two molecules in particular, Tube and Pelle, were solved in complex with each other and illustrate one distinct mode of death domain (DD) complex formation (22). As with the Toll receptor, Tube and Pelle were originally found to be involved in the development of the dorsal-ventral axis in Drosophila embryos (23, 24). Later they were found to mediate the innate immune response of Drosophila against fungal infection (21). Pelle is a serine-threonine kinase that bears an amino-terminal DD, a carboxyl-terminal kinase domain (KD), and is homologous to the mammalian IRAK family members. Tube has an amino-terminal DD but lacks a TIR domain and a mammalian counterpart. Tube also was once thought to be a functional homolog of MyD88 but later

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was found not to be the case when a TIR-domain containing Drosophila homologue was discovered to be an adaptor molecule involved in the Toll signaling pathway (25). Drosophila MyD88 (dMyD88) was found to associate directly with the cytoplasmic portion of the Toll receptor and required Tube and Pelle to activate the expression of the antifungal peptide Drosomycin (26). Although Tube does not interact directly with the Toll receptor (27–29) and its exact role in signaling is unclear, it plays a crucial role in host defense. It is apparent that Tube does bind directly to Pelle both in vivo and in vitro (22, 23).

The dissociation constant for the Pelle-DD/Tube-DD complex by a combination of different methods was calculated to be 0.5 μM (30). Pelle and Tube crystallized as a tetramer, containing two molecules of each, but whether this persists in solution has yet to be determined. However, the Pelle-DD and Tube-DD heterodimer has been demonstrated to be stable in solution for long periods of time. The complex crystal structure of the Pelle and Tube DDs revealed the canonical hexahelical bundle indicative of a DD (22) (Fig. 3A). The hexahelical bundle as a structural domain is common to the death effector domain, as well as to the caspase recruitment domain, and comprises a protein superfamilly (reviewed in Ref. 31). A conserved interaction surface in the DD, analogous to the bb loop of the TIR domain, is apparently absent, suggesting that DDs may associate by a diverse set of mechanisms (22). The association mechanism between the DDs of Pelle and Tube did indeed prove to be novel and perhaps unique to these innate immune molecules. Analysis of the complex crystal structure revealed two main points of contact between Pelle and Tube. One interaction site is the insertion of the carboxy terminus of Pelle α4 into a groove of Tube.

This interface is stabilized mainly by hydrogen bonds. Another interaction site is formed by the carboxyl-terminal tail of Tube, which lays in a groove formed by the α4-α5 and α2-α3 loops of Pelle. This second interface is stabilized by a number of hydrogen bonds and van der Waals forces. The carboxyl-terminal tail of Tube was found to be indispensable for in vivo Pelle/Tube activity to take place (22) (Fig. 3A). The tight association between the DDs of Pelle and Tube might be an important regulatory step to prevent misactivation of the Pelle kinase. Pelle is known to autophosphorylate, and this occurrence prevents association with Toll and Tube. Autophosphorylation takes place at the Pelle DD and has been proposed to occur subsequent to Pelle dimerization (29). The Pelle-DD alone has not been demonstrated to form homodimers in solution. In addition to the complex crystal structure, the crystal structure for Pelle-DD alone has been determined (32) but is severely distorted by a crystallization reagent. Instead of forming six helices folded into a compact bundle as in the complex structure, the structure of Pelle-DD alone resembles a long rod comprised of one α-helix, which dimerizes with a neighboring helix resembling a pair of scissors. Although this structure form is most likely due to a crystallographic artifact, it has been proposed that this refolding event from hexahelical bundle to a long α helix could take place in vivo in the context of cell membrane association (32). Previous examples of proteins such as colicins or cytochrome c are transformed in tertiary or even quaternary structure after insertion into or lateral contact with the membrane. This refolding event in the Pelle-DD could possibly play a role in kinase dimerization and subsequent activation (32).

The Pelle-DD crystal structure alone obviously raises some questions on the validity of the structure in general. The solution structure of the Pelle-DD was also determined by another group using NMR (33). This structure very much resembled the Pelle-DD in the complex crystal structure with Tube. In addition, interface analysis of the Pelle-DD/Tube-DD complex using solution NMR suggests that the interactions obtained in solution are similar to those found in the crystal structure validating those findings. The NMR structure of Pelle-DD alone revealed that the amino and carboxy terminal tails were quite dynamic in solution.

The DDs of Tube and Pelle interact, so does the DD of dMyD88 play any role in this signaling process? Sun et al. (34), using RNA interference experiments, demonstrated that dMyD88, Tube, and Pelle form a linear signaling hierarchy with Tube acting downstream of MyD88 but upstream of Pelle. They went on to further show that the DD of Tube is sufficient to mediate the association of full-length dMyD88 and Pelle. A surface mutation in Tube (E50K) abrogates binding of Pelle and abolishes Tube function in Toll signaling (22) but does not affect binding to dMyD88. This finding suggests that the Tube-DD has two distinct binding surfaces: one to mediate binding with Pelle (involving E50) and the other to dMyD88 (E50-independent). In light of these results, Tube, Pelle, and dMyD88 predictably form a heterotrimer, and this complex is a critical step in Toll signaling (34).

The structural role of DDs in TLR signaling

DDs are important in the proximal TLR signal transduction process (Fig. 1). MyD88 is the only adaptor molecule known to contain a DD. The DD serves to recruit a family of kinases known as IRAks. The IRAK family (reviewed in Ref. 35) has
four known members: IRAK-1 (36), IRAK-2 (37), IRAK-M (38), and IRAK-4 (39). All four IRAKs contain a DD at the N terminus and a kinase domain at the C terminus; however, only IRAK-1 (36) and IRAK-4 (39) have been shown to have kinase activity. IRAK-4 is a unique member of the IRAK family in that it is the only member to have true kinase activity (i.e., phosphorylates substrates other than itself) and it is also the most homologous to Pelle. To mediate TLR/IL-1R signaling, IRAK-4 associates with the intermediate domain (ID) of the adaptor MyD88 presumably via its own DD (2). IRAK-1, on the other hand, associates with the DD of MyD88 effectively poised for phosphorylation by IRAK-4. This phosphorylation most likely takes place on the activation loop of IRAK-1 (within the kinase domain) on residues T387 and S376 (39). Once this phosphorylation event occurs, IRAK-1 kinase activity increases and it heavily autophosphorylates residues in its amino terminus (39–41). A splice variant of MyD88 (MyD88s), missing the intermediate domain, has been shown to shut down IL-1β/LPS-induced NF-κB activation by interfering with IRAK-4 association (2). IRAK-4 strongly associates with full-length MyD88, but not with MyD88s, whereas IRAK-1 has no preferential binding partner. Also, in the presence of MyD88s, IRAK-1 is not phosphorylated or subsequently ubiquitinylated as in the normal signaling cascade (2, 42). In light of these results, MyD88s acts as a negative regulator of IL-1β/LPS-induced NF-κB activation by preventing IRAK-4’s access to its substrate. At this time, it is unclear what exact molecular determinants between MyD88 and IRAK-4 are necessary in order for interaction and subsequent signaling to occur. Yeast two-hybrid assays indicate that the DD, ID, or TIR domains of MyD88 alone are insufficient for association with full-length IRAK-4 to occur (2). However, a construct containing the entire DD, ID, and a small portion of the TIR domain of MyD88 (residues 1–172) was found to strongly associate with full-length IRAK-4 (2). The minimal portion of IRAK-4 necessary for this interaction with MyD88 to take place was not demonstrated in this set of experiments. In our laboratory, using a GST-IRAK-4 DD (residues 1–113) fusion protein and in vitro-transcribed and -translated MyD88 constructs, pulldown assays indicate that the carboxyl-terminal portion of the ID and the TIR domain (this construct excludes the DD entirely) are sufficient to mediate binding with IRAK-4 DD (unpublished observations). Taken together, these results indicate that DD-DD homotypic protein interactions might not play a role in the MyD88/IRAK-4 interaction mechanism but rather the DD of IRAK-4 possibly recognizes the ID of MyD88 (Fig. 1).

The first crystal structure of an IRAK family member, IRAK-4DD, was determined in our laboratory and reveals a hexahelical bundle indicative of a DD protein (43) (Fig. 3B). The true core structure spanned amino acid residues 4–106 and was proceeded by two conserved proline residues, P102 and P106, that might play a role in orienting the C-terminal kinase domain so that proper molecular recognition can take place (43). Functionally important residues in IRAK-1 DD (44) were found to be critical structural components of the IRAK4-DD, indicating that the little conservation that does exist within the DDs of IRAK-1 and -4 is imperative for maintaining structural integrity of the hexahedral bundle. Two strictly conserved residues, D27 and T67, tether the first and fourth helices together by means of an intimate hydrogen bond. The crucial structural role of T67, combined with previous studies (45), suggests that this site might be important for regulation of IRAK-4 (43).

IRAK-4 is the most homologous IRAK family member to Pelle. The structures of IRAK-4 and Pelle are nearly identical with the exception that the highly structured and sizeable α2-α3 loop in IRAK-4 is quite minute in Pelle. Using a primary sequence alignment of the IRAK family and Pelle, it was determined that the sizeable nature of the α2-α3 loop is unique to IRAK-4. Additional comparative analysis of the Pelle-DD/Tube-DD and IRAK-4 DD crystal structures suggests that the cognate adaptor interface present in Drosophila is not likely to be evolutionarily conserved in mouse (43). The protruding α2-α3 loop in IRAK-4 (Fig. 3B) is likely to be involved in molecular recognition because of its structural uniqueness but indeed would need to be accommodated by the interacting protein. The role of this loop in mediating downstream signaling or if there is a function for the α2-α3 loop has yet to be elucidated.

The DD of IRAK-4, although important in molecular recognition, is itself unable to sustain signaling or offer immunological protection. Patients with mutations in IRAK-4 that encode proteins with intact DDs, but truncated kinase domains, are highly susceptible to recurrent bacterial infections (5). In addition, it was demonstrated that these truncated proteins were capable of sequestering MyD88 to the cytoplasm, preventing its interaction with kinase-competent IRAK-4 molecules upon IL-1 stimulation (46). In this study, IL-1-induced IRAK-1 association with the IL-1R was shown to be reduced in the presence of truncated IRAK-4 protein. Medvedev et al. (46) also proposed that mimetics of these truncated IRAK-4 proteins may represent a way of mitigating hyperinflammatory states and therefore be useful as a potential therapeutic. As expected, humans completely deficient of any form of IRAK-4 are vulnerable to pyogenic bacterial infections (6). Disruption of IRAK-4 obviously has profound consequences on the immunological protection of humans, and this is also true of mice (47). Both human and mouse IRAK-4 deficiencies have severe impairment of IL-1 and TLR downstream signaling. When IRAK-4 knockout mice are subjected to LPS challenge, their survival is not impaired, and their phenotype is much like the P712H mutation of Tlr4. These IRAK-4 properties make it an attractive drug target. Modulation of this key mediator could prove useful in treating disease states of inflammation, septic shock, and in the management of autoimmune disorders (43).

From the TIR structures, we have learned about the cytoplasmic portion of the TLR at a molecular level and what surfaces are important for adaptor binding. We also learned more about the structural basis of the P712H mutation of Tlr4. These IRAK-4 properties provided us clues regarding the critical amino acids in the recognition domain of an important kinase involved in TLR and IL-1R signaling. This structural knowledge will hopefully not only guide future mutagenesis and characterize protein functions but serve as a starting point for the design of novel therapeutics. Although these structures have answered many questions about the molecular basis of TLR signaling countless, questions still remain unanswered. Complex crystal structures of the TIR domains of receptor and adaptor will elucidate the details of the interface and the interactions necessary for recognition to take place. A
targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to viru-

codon polymorphism abolishes flagellin signaling and is associated with susceptibility

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