Toll signaling: the enigma variations

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Experiments reported in the past year have revealed considerable diversity in Toll-mediated pathways for signal transduction in development and innate immunity. Rather than function as a well conserved signaling cassette, Toll receptors and associated factors have apparently evolved as a diverse set of configurations to defend against microbial infection in species ranging from plants to humans.

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Abbreviations

gd	*gastrulation defective*
IKK	IκB kinase
IL-1	interleukin-1
imd	*immune deficiency*
IRAK	IL-1 receptor associated kinase
Ird	*immune response deficient*
LPS	lipopolysaccharide
LRR	leucine-rich repeat
NLS	nuclear localization signal
Tlr	Toll-like receptor

Prelude

The Toll signaling pathway was discovered independently in the biochemical investigation of cytokine responses in cultured mammalian cells and in the molecular genetic dissection of embryonic patterning in *Drosophila* [1–3]. In both systems, transmembrane receptors of the Toll family signal through adaptor molecules and protein kinases to effect nuclear localization of a transcription factor (Figure 1). Evolutionary conservation of the systems first became apparent with the discovery that the mammalian transcription factor NF-κB and the *Drosophila* morphogen Dorsal, the targets of these pathways, were both homologous to the products of the vertebrate proto-oncogene c-*rel*.

With the characterization of additional components and the elaboration of more mechanistic details, it appeared that the Toll-family receptors and associated factors constituted a conserved signaling cassette employed for distinct purposes at different times and in different tissues. The Toll pathway would thus take its place alongside other conserved signaling systems, such as those described for Wnt and Ras [4,5]. Recent evidence, however, indicates that there are in fact several different Toll pathways. These pathways carry out signal transduction by distinct routes, yet are largely restricted to a single function, that of defense against infection.

One Toll pathway involves direct activation of the receptor by a pathogen-encoded macromolecule. A second functions as part of a signal relay system, mediating intracellular signaling in response to activation of a host-encoded ligand. Others involve a limited subset of pathway components. Together, these divergent forms of the Toll-signaling cassette constitute an intriguing series of variations based on a common molecular and functional theme. Here I review these variations, bringing into a common context recent results from biochemical experiments, genetic studies, and genome analyses.

Statement of the theme: Toll-like receptors in mammalian innate immunity

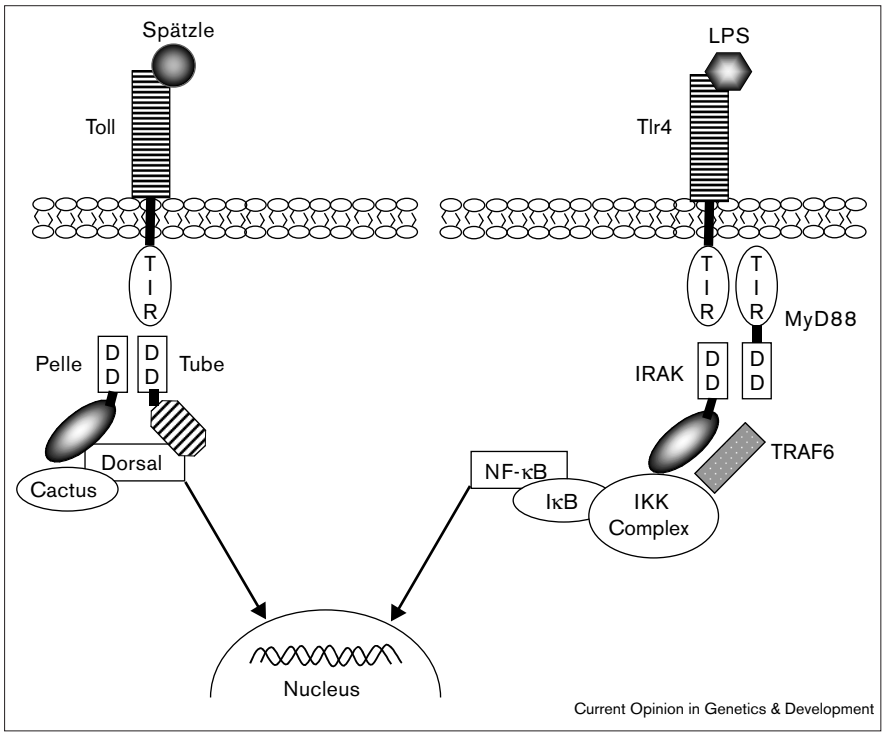
Upon binding with bacterial lipopolysaccharide (LPS) or interleukin-1 (IL-1), mammalian cell-surface receptors initiate a signal-transduction pathway that directs release of NF-κB (a p50/p65 heterodimer) from its inhibitor IκB (Figure 1b) [6]. Receptor activation drives formation of a complex that includes the IL-1 receptor associated kinase (IRAK) and two adaptor proteins, TRAF6 and MyD88. This multiprotein assembly mediates activation of the IκB kinase (IKK) complex, which targets IκB for degradation via phosphorylation of specific serine residues [7•]. The subsequent ubiquitination and proteolysis of IκB exposes the nuclear localization signal (NLS) on NF-κB, allowing nuclear import and activation of gene expression.

In the innate immune response to infection, the critical receptors for NF-κB regulation are homologues of the *Drosophila* Toll protein. Toll and Toll-like receptors (Tlrs) from flies and mammals contain extracellular binding surfaces comprising leucine-rich repeats (LRRs), a single membrane-spanning domain, and an intracellular domain that mediates signal transduction but lacks apparent catalytic activity [8,9]. Conservation between the Toll cytoplasmic domain and that of both the IL-1 receptor and some plant *R* (disease resistance) proteins led to the designation of this region as the TIR domain.

The demonstration that a transfected Tlr gene could activate NF-κB and the identification of *Tlr4* mutations in LPS-resistant mice established Tlr4 as a critical mediator of LPS signaling [10,11]. Moreover, the Tlr4 gene from a given mammal (human, mouse, or hamster) confers on transfected cells the ability to respond to the specific LPS derivative to which that species is sensitive [12••,13••]. Such results suggest strongly that LPS interacts directly with Tlr4 to activate signal transduction.

Given the existence of more than a half dozen mammalian Tlr genes, there is the potential for different family members to be specific for different sets of microbial pathogens [14•]. Indeed, several studies reveal that Tlr2 and Tlr4 recognize distinct microbial products: Tlr2 is specific for Gram-positive bacteria, whereas Tlr4 recognizes Gram-negative species

Figure 1



Variations on the Toll Pathway. **(a)** Dorsoventral axis formation in *Drosophila* embryos. A cleaved form of Spätzle generated in the ventral portion of the extraembryonic space binds to and activates Toll. The Toll cytoplasmic domain (TIR) mediates signaling to a complex of Cactus and a Dorsal dimer. The detailed mechanism is not known but requires interaction between the death domains (DD) of Pelle and Tube, as well as the catalytic activity of the Pelle kinase domain. Signaling to Cactus and to Dorsal results in Cactus degradation and Dorsal nuclear import. The pathway for the antifungal response appears identical, except that Dif serves in place of Dorsal. **(b)** Innate immune response in human cells. Binding of LPS to the Tlr4 receptor leads to interaction between the TIR domains of Tlr4 and MyD88. A complex of IRAK and TRAF6, formed upon interaction of the MyD88 and IRAK death domains, activates the IKK complex. This assembly of the IKKα and IKKβ kinases and the IKKγ (NEMO) regulatory subunit phosphorylates serines 32 and 36 on IκB-α. The subsequent ubiquitination and proteasome-mediated degradation of IκB exposes the NLS on NF-κB, promoting nuclear translocation.

[13••,15••,16••]. Hence, for example, mice lacking a functional copy of the *Tlr2* gene respond normally to LPS but are defective in their response to peptidoglycan. The Tlr molecules may, thus, form recognition elements that prime the immune system for battle with particular classes of infectious agents [17].

Variation 1: Toll signaling in antifungal defense and pattern formation

The Toll pathway in *Drosophila* was first identified on the basis of its role in establishing embryonic dorsoventral polarity (Figure 1a) [2]. Homodimers of the Dorsal protein are initially present throughout the embryonic cytoplasm, where they are retained by an inhibitor, Cactus. Following fertilization, a localized source of the ligand Spätzle is believed to activate Toll in a graded ventral to dorsal pattern over the surface of the syncytial embryo. Activated Toll signals to the Dorsal/Cactus complex via an adaptor, Tube, and a protein kinase, Pelle. The result is Cactus degradation and formation of a nuclear concentration gradient of Dorsal that defines dorsoventral polarity.

Spätzle, Toll, Tube, Pelle, and Cactus function in concert again in both larvae and adults as part of the invertebrate innate immune response [18]. Upon fungal challenge, wild-type *Drosophila* express Drosomycin, a potent antifungal peptide. This immune response is protective, because flies mutant for *spätzle*, *Toll*, *tube*, *pelle*, or *cactus* fail to induce the *drosomycin* gene and succumb to fungal infection much more readily than the wild type. The direct inducer of *drosomycin* in adults is not Dorsal, but rather Dif, the *Drosophila* immunity factor [19••].

Despite the fact that Toll, Pelle, Cactus, Dorsal and Dif all have structural and functional counterparts in vertebrates, the Dorsal/Dif pathway differs from the NF-κB pathway in several significant respects. First, Spätzle, the Toll ligand, is not a fungal product but is instead a host-encoded protein that is activated by proteolysis [20,21••]. In dorsoventral patterning, Spätzle is activated by a proteolytic cascade involving the products of the *gastrulation defective* (*gd*), *snake*, and *easter* genes [22]. A different proteolytic pathway is required in the antifungal defense because mutations in a serine protease inhibitor (serpin) gene result in constitutive Drosomycin expression even in the absence of *gd* or *snake* function [21••]. Thus it appears that fungal infection triggers a proteolytic pathway leading to activation of Toll, which then relays information to the nucleus via Dif.

A second way in which the *Drosophila* Toll pathway differs from the mammalian Tlr4 pathway is in the proteins required to induce Cactus degradation (Table 1). Cactus proteolysis is regulated, at least in part, by motifs similar to those targeted by vertebrate IKK proteins and by a conserved pathway for signal-induced ubiquitination [23,24,25•]. Nevertheless, the *Drosophila* homologues of the kinase IKKβ and the regulatory subunit IKKγ are dispensable for dorsoventral patterning and the anti-fungal response (J Hoffmann, personal communication; K Anderson, person-

al communication). A remaining candidate for the Cactus kinase is DmIKKε, for which mutations have not been described. DmIKKε is a counterpart to IKKε and related vertebrate kinases, which transduce signals to NF-κB in T-cell activation and perhaps other processes [26•–29•].

The Dorsal/Dif pathway also differs from the NF-κB pathway in the adaptor proteins that act downstream of the receptor. MyD88 contains a TIR domain that interacts with the TIR domain of Toll family receptors, whereas Tube protein has a novel repeat that mediates binding to Dorsal [30–32]. Furthermore, although MyD88 and Tube interact with IRAK and Pelle, respectively, via interaction motifs termed death domains [33,34,35••], the binding of Tube to Pelle involves sequences outside the death domain that are not conserved in either MyD88 or IRAK [35••].

A remaining difference observed in the *Drosophila* system is an apparent bifurcation in the dorsoventral pathway, such that there is signaling from Toll, Tube, and Pelle not only to Cactus but also to Dorsal. Specifically, a nuclear concentration gradient of Dorsal persists, albeit in an attenuated form, in the absence of either Cactus or a Dorsal–Cactus interaction [24,36•]. This residual gradient might reflect regulation of Dorsal by Relish, the only *Drosophila* protein other than Cactus with an IκB-like domain (see below). It seems more likely, however, that the observed signal-dependent phosphorylation of Dorsal and the binding of Pelle to Dorsal in embryos reflect a pathway for Cactus-independent signal transduction to Dorsal [31,37,38].

Variation 2: *Drosophila* anti-bacterial responses

Drosophila mount an immune response not only against fungi but also against bacteria, inducing expression of anti-bacterial peptides such as Diptericin, Attacin, and Cecropin [39•]. The pathway leading to Diptericin induction is distinct from the anti-fungal pathway because it is unaffected by mutations in *dorsal*, *Dif*, or the genes that regulate these factors [18,40•]. Furthermore, a number of genes specific for this pathway have been identified, including the *imd* (*immune deficiency*) locus and the *ird* (*immune response deficient*) genes [39•,41,42] (Table 1).

The target of the *imd/ird* pathway is Relish, a p105 homologue active in humoral immunity [43••]. Like the mammalian p105 protein, Relish contains both a Rel homology domain and a set of IκB-like ankyrin repeats. A null mutation in *relish* renders flies highly susceptible to bacterial infection and eliminates induction of *diptericin* in response to infection. Surprisingly, processing of Relish is quite different from that of p105. Whereas signaling in mammalian cells leads to proteasome-mediated cleavage of p105 and degradation of the IκB-like domain [44], signal-dependent cleavage of Relish is proteasome-independent and results in stable Rel and IκB-like fragments (D Hultmark, personal communication).

Table 1

Components of Toll signaling pathways in *Drosophila*.

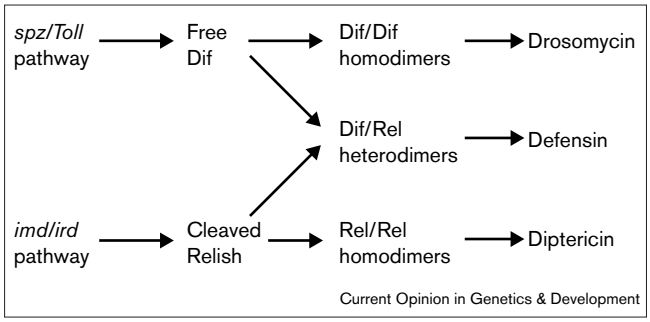
	Antifungal and Dorsoventral	Antibacterial (Diptericin)	Accession nos.†/ other names
Receptors			
Toll	+	–	
18-wheeler	–	+	
Toll-3			MstProx
Toll-4			CG18241
Toll-5			Tehao
Toll-6			CG7250
Toll-7			CG8595
Toll-8			Tollo
Adaptors			
Tube	+	–	
DmMyd88			CG2078
dTraf1			
dTraf2			
Kinases			
Pelle	+	–	
DmIKKβ	–	+	Ird-5, dLak, Ik
DmIKKγ	–	+	Kenny
DmIKKε			Ik2
Effectors/inhibitors			
Cactus	+	–	
Dorsal	+*	–	
Dif	+*	–	
Relish	–	+	

For each gene for which mutations have been characterized, symbols indicate whether the gene is required (+) or not (–) for the pathways listed. *Dif is required for the antifungal pathway in adults; Dorsal is required for the dorsoventral pathway in embryos. These two loci are redundant for the antifungal response in larvae [40•]. †The accession numbers and alternate names are archived in Flybase (http://flybase.bio.indiana.edu/).

DmIKKβ and DmIKKγ, dispensable in fighting fungal infection, are both active in the anti-bacterial immune response. Mutations in either locus block Diptericin induction but have no effect on Drosomycin expression (J Hoffmann, personal communication; K Anderson, personal communication). Furthermore, an activated complex of DmIKKβ and DmIKKγ phosphorylates Relish *in vitro* (N Silverman, D Hultmark, T Maniatis, personal communication). Given the involvement of an IKK complex, it seems likely that the Diptericin pathway will also involve the *Drosophila* homologues of TRAF6 [45,46] and MyD88 (S Wasserman, unpublished data).

At least one Tlr protein, 18-Wheeler, is expressed in the larval fat body, the focus of the humoral immune response, and appears to be active in the *Drosophila* antibacterial response [47]; however, because *diptericin* expression is relatively unaffected in an *18-wheeler* mutant, it is likely that further genetic analyses will place additional Tlr proteins in the *imd/ird* pathway. Given that *Drosophila* encodes a repertoire of eight Toll family receptors (see Table 1), there is the potential, as in mammals, for substantial specificity in pathway activation and function.

Figure 2



Model for combinatorial control of antimicrobial genes in *Drosophila*. Homo- and heterodimers of Dif and the amino-terminal domain of Relish differ in their affinity for the κB sites governing expression of the loci encoding Drosomycin, Defensin, and Diptericin. As a result, activation of the *spätz/eToll* and *imd/ird* pathways singly or in combination provides a means for the independent regulation of these three antimicrobial genes. If Dorsal as well as additional Tlr pathways are included in the scheme, up to six sets of such genes can be differentially regulated.

Although the *imd/ird* pathway of *Drosophila* bears substantial similarity to that of mammals, the mechanism of signal transduction is most likely different. In the NF-κB pathway, the IRAK kinases are necessary for signal transduction [48••,49]. In the *imd/ird* pathway, however, Pelle, the only IRAK ortholog in flies, is not required for Diptericin induction.

Unlike the *diptericin* gene, for which the *imd/ird* pathway is both necessary and sufficient to achieve activation, most *Drosophila* antibacterial genes require more than one input for full induction [39•]. For example, both the *spätzle/Toll* and *imd/ird* pathways are required to induce the *defensin* gene. As transfection of different combinations of Dorsal, Dif, and Relish drives expression of distinct sets of immune factors [50], it seems likely that signals from particular Toll pathways, alone or in combination, activate specific homo- and heterodimers of the three *Drosophila* Rel proteins (Figure 2).

Further variations

The extraordinary breadth of Toll pathway conservation became apparent when it was found that the *N* gene of tobacco was related in sequence to Toll and that many additional plant disease resistance (*R*) genes encode Pelle homologues, such as Pto [51,52]. The basis of this conservation was unclear at first because the *N* gene product and other plant Toll homologs were predicted to be cytoplasmic, containing LRR repeats and TIR domains, but no transmembrane domain. The question as to how such proteins function in pathogen recognition was answered with the finding that both viruses and bacteria introduce pathogenic gene products directly into plant-cell cytoplasm [53].

Given that no plant Rel homologues have been identified, the plant LRR–TIR and Pelle-like *R* proteins most likely function in signaling cascades quite divergent from their *Drosophila* and human counterparts. Indeed, to the extent that there is any evidence ordering these genes in a pathway, the Pelle-like kinases appear to act upstream of the LRR–TIR proteins [54••]. Consistent with such an order of action, Pto appears to participate directly in pathogen recognition, binding to the *Pseudomonas* avrPto protein via activation loop sequences that are essential for *Pseudomonas* resistance [52].

Like plants, the nematode *Caenorhabditis elegans* appears to encode Toll pathway components but no Rel-like proteins [55]. *C. elegans* also differs from other animals in having only a single Tlr gene, in addition to single genes belonging to the Pelle, IκB, and TRAF families. As at least three of these four genes are dispensable for normal morphological development, the pathway has been presumed to function in immunity. Assays with known pathogens, however, have not revealed an immune deficiency in worms lacking the function of any of these loci.

Recent studies in both mammals and flies have identified several new proteins in Toll signaling pathways. The Tollip protein in mammals binds to IRAK and apparently acts to inhibit signal transduction prior to cytokine stimulation [56•]; a readily recognizable homolog exists in worms, but not flies. In contrast, a mammalian TRAF6 interactor termed ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) has a *Drosophila* homologue which binds to dTRAF1, the fly TRAF6 counterpart [57•]. Furthermore, dTRAF1 also interacts with Pelle, which binds not only to Tube and Dorsal, but also to Toll and Pellino, a novel fly protein [46,58,59]. There are, thus, clearly additional networks of interactions that need to be sorted out.

Coda: perspectives

In recent years, we have grown used to conservation in mechanistic detail in concert with functional diversity. The Wingless and Ras signaling cassettes are highly conserved at the biochemical level but function in a diverse set of developmental processes [4,5]. In the case of the Tlr pathways, the opposite is true: function is largely constant but the pathways have diverged. The contrast is apparent even at the level of individual components — sequence identity between *Drosophila* and human components of the Toll pathway is in general much lower than that of components in the Wingless or Ras pathway.

Conserved function and diverged mechanism makes sense for a pathway that evolved to carry out immune function. Distinct pathogens present distinct problems for infected organisms, synthesizing diverse sets of macromolecules and disrupting growth and metabolism by disparate routes. In this context, variations in the Toll-signaling pathway could be means of linking specific ligands to specific responses. How such immune pathways were co-opted for development in *Drosophila*, and perhaps other organisms [60], remains an open and fascinating evolutionary question.

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