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Conventional and non-conventional *Drosophila* Toll signaling

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ABSTRACT

The discovery of Toll in *Drosophila* and of the remarkable conservation in pathway composition and organization catalyzed a transformation in our understanding of innate immune recognition and response. At the center of that picture is a cascade of interactions in which specific microbial cues activate Toll receptors, which then transmit signals driving transcription factor nuclear localization and activity. Experiments gave substance to the vision of pattern recognition receptors, linked phenomena in development, gene regulation, and immunity into a coherent whole, and revealed a rich set of variations for identifying non-self and responding effectively. More recently, research in *Drosophila* has illuminated the positive and negative regulation of Toll activation, the organization of signaling events at and beneath membranes, the sorting of information flow, and the existence of non-conventional signaling via Toll-related receptors. Here, we provide an overview of the Toll pathway of flies and highlight these ongoing realms of research.

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1. Toll pathway conservation and divergence

In the face of assault from bacteria, viruses, fungi, and parasites, multicellular organisms defend themselves. Receptors that respond to conserved microbial molecules trigger signaling cascades that direct expression of a battery of antimicrobial peptides, cytokines, and other immune mediators. In both vertebrates and invertebrates, signaling by transmembrane Toll receptors has a central role in these responses (Brennan and Anderson, 2004; Imler et al., 2004; Kawai and Akira, 2011; Kim and Kim, 2005; Pasare and Medzhitov, 2005). Indeed, the pathways mediated by Toll and Toll-like receptors (TLRs) exhibit striking evolutionary conservation, as shown in Fig. 1.

The Toll pathway of *Drosophila melanogaster* was first described in the context of the syncytial blastoderm embryo. There it establishes the dorsoventral axis by regulating nuclear localization of the transcription factor Dorsal (Anderson and Nusslein, 1984; Nüsslein-Volhard et al., 1987; Steward, 1987). Prior to Toll activation, the inhibitor protein Cactus retains Dorsal in the cytoplasm by masking its nuclear localization signal (NLS). Toll signaling, which relieves this inhibition, is triggered by a proteolytically activated form of the Spätzle (Spz) protein (Schneider et al., 1994). Cleaved Spätzle binds to Toll, triggering a conformational change that generates an active Toll dimer. The dimerized cytoplasmic domain of Toll interacts with an adaptor, MyD88, which recruits a second adaptor Tube and the protein kinase Pelle. This signaling complex initiates phosphorylation and degradation of Cactus, freeing Dorsal to enter nuclei (Belvin and Anderson, 1996; Drier and Steward, 1997; Wasserman, 2000). Because Toll signaling is spatially graded across the syncytial embryo, the result is a nuclear concentration gradient of Dorsal that elicits broad stripes of dorsal, lateral, and ventral gene expression (Ip et al., 1991; Kanodia et al., 2009; Stathopoulos et al., 2002; Stein and Stevens, 1991).

The Toll pathway mediating *Drosophila* innate immunity has the same architecture as that directing embryonic axis formation. Innate immune function, however, involves an alternative transcriptional factor, the Dorsal-related immunity factor (Dif) (Ip et al., 1993). Dorsal and Dif overlap in function in larvae, with either being sufficient for immune function (Ip et al., 1993; Lemaitre et al., 1995b; Manfruelli et al., 1999). In adults, only Dif is required (Meng et al., 1999).

Beginning with Toll, each component of the *Drosophila* pathway has a mammalian ortholog (see Fig. 1). Thus, for example, the fly proteins MyD88, Tube, and Pelle have direct counterparts in mammalian MyD88, IRAK4, and IRAK1. Each of these proteins contains a death domain, a protein interaction motif first described in apoptotic pathways (Tartaglia et al., 1993). Similarly, the fly proteins Dorsal and Dif are similar in sequence and function to the mammalian NF-κB proteins. Each contains a Rel homology region, a conserved protein domain that has sites for DNA binding, for dimerization, and for interaction with an inhibitor. That inhibitor – Cactus or its ortholog IB – has N-terminal sites for signal responsiveness, ankyrin repeats that bind Rel proteins, and a destabilizing C-terminal PEST domain.

Although each pathway component in flies has a counterpart in mammals, the converse is not true. TLR signaling to NF-κB and IκB requires a number of components not found in the *Drosophila* Toll

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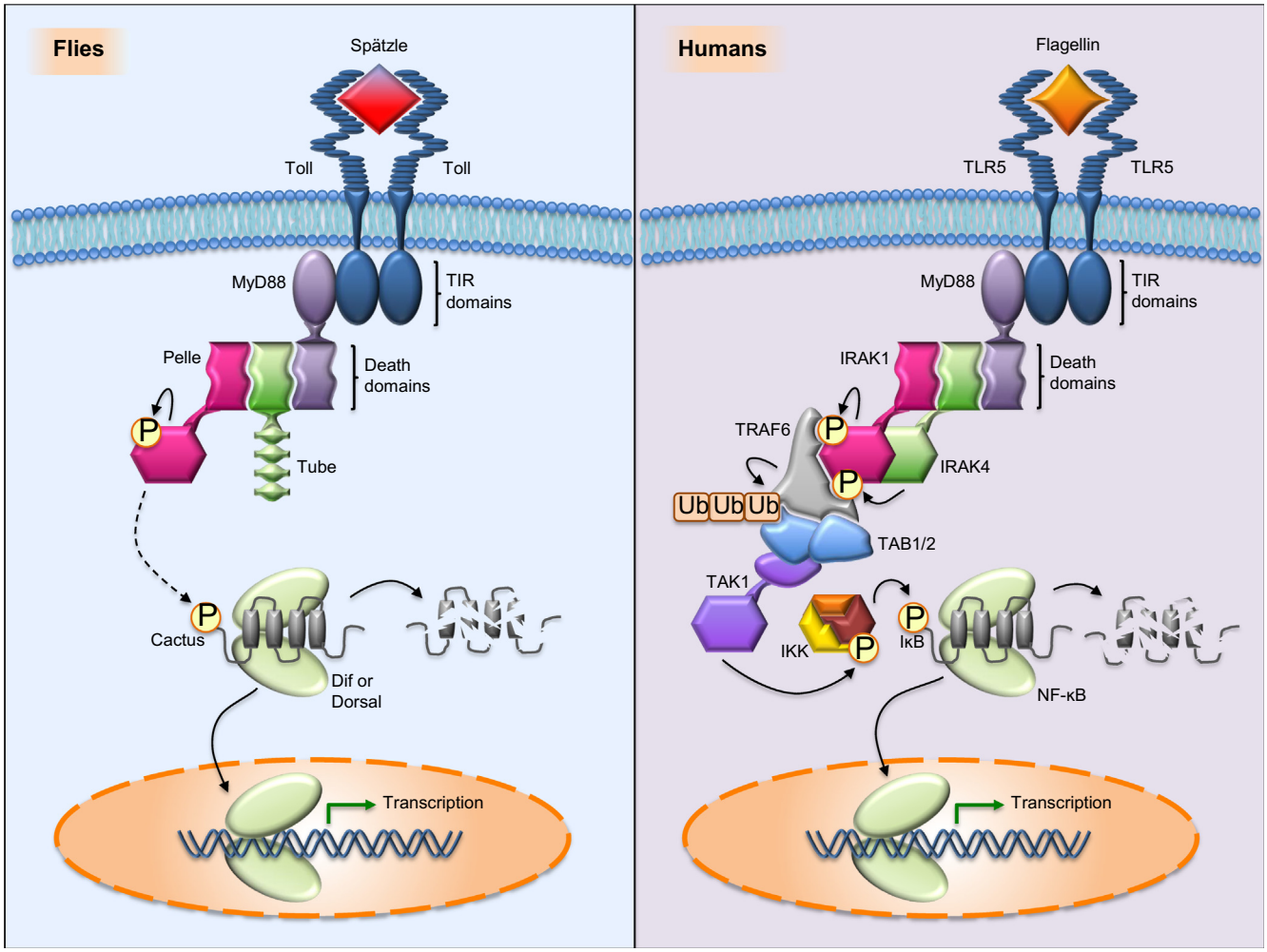

Fig. 1. Evolutionary conservation of the *Drosophila* Toll and human TLR signaling pathways. (Left) in flies, Toll signaling is activated when a processed form of Spätzle binds the Toll ectodomain. Toll activation triggers dimerization of the intracytoplasmic TIR domains, which promotes binding of the adaptor protein MyD88 through its own TIR domain. MyD88 binds the adaptor protein Tube, which in turn recruits the protein kinase Pelle, each interaction occurring via pairwise interaction of death domains. Although only one signaling module is shown, each TIR domain of the Toll dimer is capable of binding one molecule of MyD88 and there are thus two signaling modules per Toll dimer. Recruitment of Pelle induces its autophosphorylation, triggering phosphorylation and destruction of the inhibitor Cactus. The transcription factor, either Dif or Dorsal depending on the context, is then freed for nuclear translocation. (Right) in humans, there are numerous TLR pathways involving often distinct but sometimes overlapping sets of PAMPs, signaling components, and transcription factors. In the example illustrated, TLR5 signaling is activated by Flagellin, a principal component of bacterial flagella. In a manner analogous to *Drosophila* Toll signaling, human MyD88 builds a signaling complex with the Tube ortholog, IRAK4, and the Pelle ortholog, IRAK1. The complex is much bigger than the *Drosophila* counterpart, comprising six MyD88, four IRAK4, and four IRAK1 molecules in a complete signaling unit. IRAK4 phosphorylates IRAK1, triggering IRAK1 autophosphorylation and dissociation from the complex. Activated IRAK1 binds TRAF6, which then autoubiquitinates and binds the TAB/TAK1 proteins. TAK1 becomes activated and phosphorylates the IKK complex, which then phosphorylates the inhibitor IκB, leading to its degradation and the nuclear translocation of NF-κB.

pathway. These include the TRAF6, TAB, and TAK1 proteins, as well as the proteins that make up the IκB kinase (IKK) complex (Chen and Chen, 2013; Karin and Delhase, 2000). Many of these signaling proteins are, however, found in *Drosophila* and function in a second innate immune pathway termed Imd and discussed below.

The amenability of *Drosophila* to genetic, molecular, biochemical, and physiological studies has fueled a steady stream of contributions to the Toll field over the last two decades (Dionne and Schneider, 2002; Ganesan et al., 2011; Govind, 2008; Lemaitre and Hoffmann, 2007; Valanne et al., 2011). In this review we provide an overview of Toll signaling in innate immunity and highlight a few of the most exciting recent developments in this area.

2. Fly Toll signaling mechanism

The initiating event for Toll signaling is cleavage of Spätzle and the binding of the C-terminal fragment to the leucine-rich repeats (LRR) of Toll (Weber et al., 2007). Binding of the Spz fragment to the Toll LRRs induces a conformational change, generating an active form of the Toll dimer (Gangloff et al., 2008). Recent studies have demonstrated that endocytosis is required for signaling by the activated Toll receptor in both dorsoventral patterning and in innate immunity (Huang et al., 2010; Lund et al., 2010). *Drosophila* Toll signaling is therefore now believed to take place at endosomal membranes, as is true for mammalian TLR3, TLR7, TLR8, and TLR9 (Kawai and Akira, 2011).

Once activated, Toll signals via a cytoplasmic TIR domain, which forms a homotypic interaction with the TIR domain of MyD88 (Horng and Medzhitov, 2001; Tauszig-Delamasure et al., 2002). Where is MyD88 prior to signaling? In flies, as in mammals, MyD88 appears to be localized at the membrane before any interaction with Toll (Kagan and Medzhitov, 2006; Sun et al., 2004). In mammals, the MyD88 adaptor-like protein (Mal) effects MyD88 localization by interacting both with MyD88 and with regions of the membrane enriched for phosphatidylinositol 4,5-bisphosphate (PIP2). In flies, a C-terminal domain of MyD88 itself interacts with

phosphoinositides and provides membrane localization essential for wild-type Toll signaling in both immunity and development (Marek and Kagan, 2012).

Tube, Pelle, and MyD88 interact via specific pairwise interactions of their death domains to form a submembranous signaling complex (Moncrieffe et al., 2008; Sun et al., 2002, 2004; Towb et al., 1998). The Tube death domain is bivalent, interacting with the death domain of MyD88 on one surface and with that of Pelle on the other. With the demonstration that Tube is the fly IRAK4 ortholog, it was hypothesized that the architecture of the fly death domain oligomer was likely to be conserved in the mammalian pathway (Towb et al., 2009). Biophysical studies confirmed this prediction, with the physical arrangement of IRAK4 and IRAK2 in the mammalian structure matching that of co-crystallized Tube and Pelle in a previous X-ray study (Lin et al., 2010; Motshwene et al., 2009; Xiao et al., 1999). Whereas the fly death domains appear to form a simple ternary complex, those in the mammalian pathway form a helical tower involving a total of 14 polypeptides (Lin et al., 2010; Wasserman, 2010).

As highlighted in Fig. 1, the parallels between fly and mammalian Toll signaling end abruptly downstream of the death domain complex. The mammalian pathway is rich with additional adaptors and kinases, culminating in phosphorylation of IκB by the IKK complex. In contrast, the *Drosophila* pathway appears to skip directly from Pelle to Cactus phosphorylation. Might there be additional kinases that were missed in the original genetic screens defining the pathway? Perhaps. However, recent large-scale RNAi screens in three separate laboratories have failed to identify any kinase other than Pelle that functionally links Toll to Cactus (Huang et al., 2010; Kuttenkeuler et al., 2010; Valanne et al., 2010). The simplest explanation is that Pelle is in fact the Cactus kinase, a hypothesis currently under active investigation.

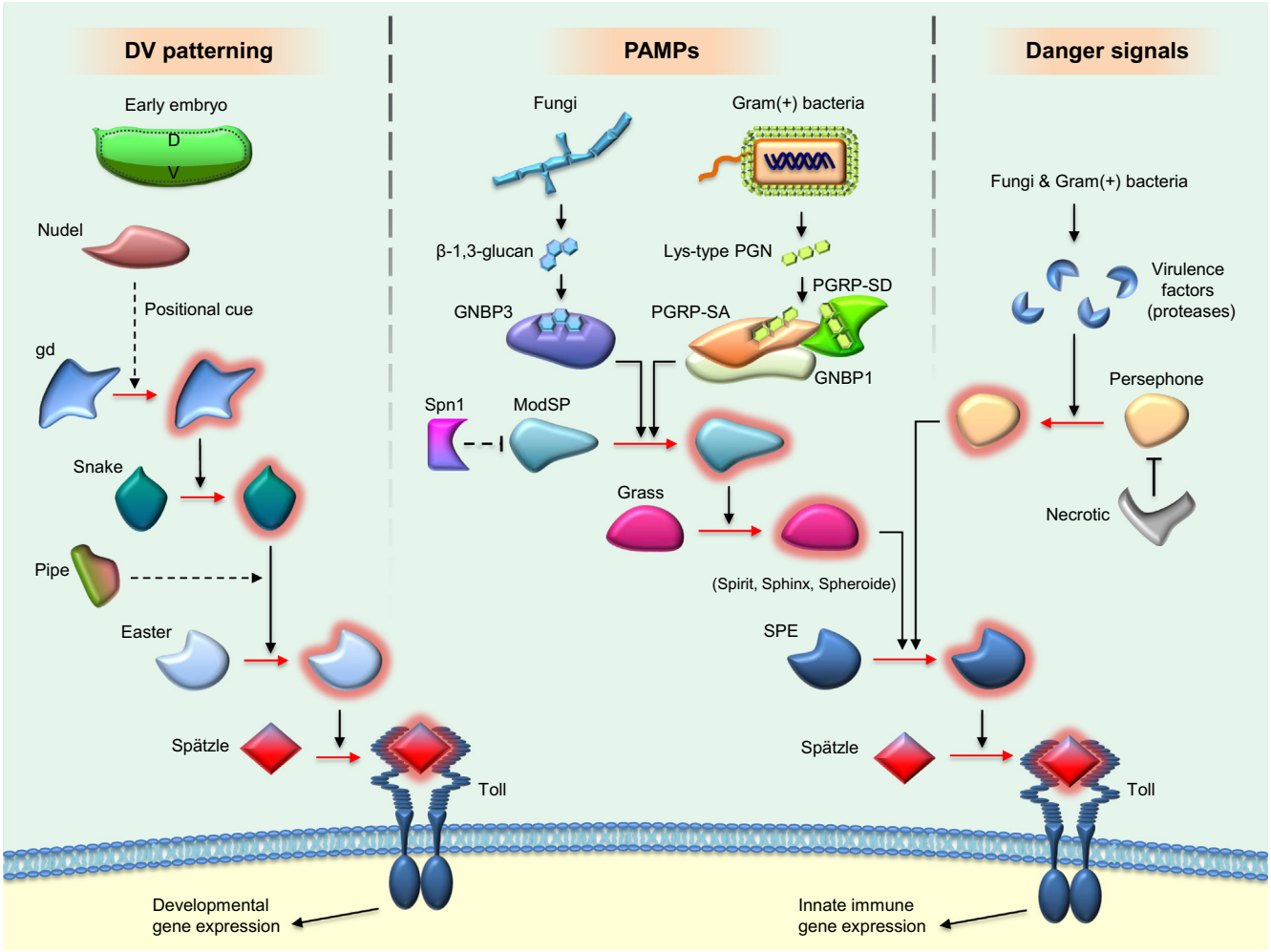

Fig. 2. Protease cascades leading to Toll activation. The active form of the Toll ligand Spätzle results from a specific cleavage triggered by any of four serine protease cascades. In these illustrations, horizontal red arrows denote proteolytic conversion of the zymogens to their active forms and a reddish glow denotes the active form of a protease. (Left) in early embryogenesis, positional cues laid out during oogenesis establish the dorsoventral axis through the localized activation of Toll on the ventral side of the embryo. The protease cascade that triggers this Toll activation involves Nudel, Gastrulation defective (gd), Snake, and Easter. Nudel directly or indirectly activates the Gastrulation defective protease, which then activates Snake. With the involvement of the sulfotransferase Pipe, activated Snake cleaves and activates Easter. Activated Easter processes Spätzle, completing generation of a functional ligand for Toll. (Middle, right) a similar mechanism operates in innate immunity, where three protease cascades converge at the activation step for the Spätzle processing enzyme (SPE). In the case of fungi and Gram-positive bacteria, the cell wall components β-1,3-glucan and Lys-type peptidoglycan, respectively, are recognized by circulating pathogen recognition receptors and trigger separate, but related protease cascades. The serine protease ModSP integrates signals from these recognition molecules and activates the protease Grass, which activates SPE. Other immune factors, such as the serine proteases Spirit, Sphinx, and Spheroide may function between Grass and SPE. In addition to recognizing PAMPs, the innate immune system is capable of sensing fungi and bacteria via the zymogen Persephone. Virulence factors (proteases) secreted from microbes cleave Persephone, resulting in activation of SPE. At several points in the pathways shown, serpins are known to provide negative regulation of these immune protease cascades. Necrotic inhibits Persephone and Spn1 inhibits upstream of Grass, with ModSP a likely target.

3. Toll activation in *Drosophila* immunity

3.1. PAMPs

Once microbes cross chemical and physical barriers to infection, host defense relies on non-self recognition. As first hypothesized by Janeway, pathogen recognition receptors (PRRs) detect conserved portions of microbes termed pathogen-associated molecular patterns (PAMPs) (Janeway, 1992). In humans, the ten TLRs function as PRRs, binding specifically to PAMPs of bacteria, fungi, and viruses. These PAMPs include bacterial flagellin, the lipopolysaccharide (LPS) of Gram-negative bacteria, and yeast zymosan (Kawai and Akira, 2011).

Fungi and bacteria activate the Toll pathway of flies. However, unlike mammalian TLRs, Toll is not a pattern recognition receptor. Rather, secreted immune factors act as PRRs and initiate proteolytic cascades that activate the Toll ligand Spätzle (Gobert et al., 2003; Leulier et al., 2003; Ligoxygakis et al., 2002b).

For fungi, it is the β-1,3-glucans (polymers of D-glucose) of cell walls that serve as PAMPs (Fig. 2). The corresponding PRR is GNBP3, a member of the Glucan-binding protein (GNBP) family. Circulating GNBP3 binds specifically to β-1,3-glucans and triggers Toll activation (Gottar et al., 2006). Inactivating the *GNBP3* locus dramatically decreases resistance to infection by yeasts such as *Candida albicans* and *Beauveria bassiana*, but has no effect on resistance to bacterial infection. In functioning as a PRR for fungi, GNBP3 also has Toll independent functions, including activation of the defensive enzyme phenoloxidase and initiation of attack complexes that target invading microbes (Matskevich et al., 2010).

For bacteria, peptidoglycan (PGN) acts as the PAMP in *Drosophila*. PGN, a major bacterial cell wall component, is a polymer assembled from alternating *N*-acetylmuramic acid and *N*-acetylglucosamine subunits. Short stem peptides cross-link the sugar chains and exhibit sequence variation characteristic of broad groups of bacteria. The Toll pathway is activated specifically by PGN in which lysine occupies the third position of the stem peptide. This Lys-type PGN is found in most, but not all, Gram-positive bacteria. Recognition involves the combined activity of two peptidoglycan recognition proteins (PGRP-SA and PGRP-SD) and a GNBP family member, GNBP1 (Bischoff et al., 2004; Buchon et al., 2009; Gobert et al., 2003; Michel et al., 2001).

Downstream of recognition, the signaling pathways that respond to β-1,3-glucans and Lys-type PGN merge. Binding of recognition proteins to either class of PAMP triggers activation of the modular serine protease (ModSP) (Buchon et al., 2009). ModSP in turn activates another serine protease, Grass (El Chamy et al., 2008; Kambris et al., 2006). The cascade continues with activation of Spätzle processing enzyme (SPE), which cleaves Spz, generating a functional Toll ligand (Jang et al., 2006). RNAi-based experiments indicate that the pathway linking Grass to SPE likely involves additional serine protease family members, including Spirit (serine protease immune response integrator), Spheroide, and Sphinx (the Sphinx1 and Sphinx2 proteins) (Kambris et al., 2006).

As diagrammed in Fig. 2, there is a marked parallel between innate immune responses and dorsoventral axis formation in the proteolytic processing of Spätzle to activate Toll signaling. From this perspective, SPE occupies the same position in the hierarchy as Easter, the terminal serine protease in the extracellular cascade in dorsoventral patterning (Chasan and Anderson, 1989; DeLotto and DeLotto, 1998). SPE has 44% overall amino acid identity with Easter and the two enzymes act at the identical site in Spätzle – 106 residues from the C-terminus (Jang et al., 2006).

Conservation between Toll activation in immunity and development is not limited to processing of Spätzle. Both pathways require proteases that contain a clip domain. This motif, first identified in a pro-clotting enzyme of the horseshoe crab, plays a central role in cascades of sequential protease activation (Muta et al., 1990; Piao et al., 2005). The immune factors Grass, Spirit, and SPE each contain a clip domain, as do the developmental factors Easter and Snake (see Fig. 2).

3.2. Danger signals

PAMP-based recognition has a shortcoming: it is ill suited for distinguishing between commensal and pathogenic microbes within body fluids. One evolutionary solution to this problem is the detection of danger signals such as virulence factors and, in some cases, their sequelae – molecules released upon the disruption of host epithelial or cellular integrity.

The Toll pathway is now known to recognize danger signals in addition to PAMPs. The particular danger signals that activate the fly Toll pathway are secreted proteases of fungi and bacteria (El Chamy et al., 2008; Gottar et al., 2006). Microbes use such proteases to degrade adherence junctions, enabling penetration of the epithelial barrier. Proteases are also key to the virulent attack of *Drosophila* and other insects by entomopathogenic fungi, which invade the insect body by boring through the insect cuticle. The protease Pr1, for example, has a crucial role in this invasive process by the fungi *B. bassiana* and *Metarhizium anisopliae*.

Detection of protease danger signals in *Drosophila* relies on Persephone (Psh), itself a serine protease (Ligoxygakis et al., 2002b). The interaction of the zymogen form of Persephone with microbial proteases that have subtilisin-like activity leads to cleavage and activation of Persephone. Once activated, Persephone directly cleaves and activates SPE, as shown in Fig. 2.

Some Gram-negative bacteria, including those belonging to the genera *Pseudomonas* and *Serratia*, secrete proteolytic enzymes. It may be that Persephone also detects these proteases and that this detection underlies the limited, but detectable, responsiveness of the Toll pathway to particular Gram-negative species (El Chamy et al., 2008).

Persephone-mediated detection of danger signals and the recognition and response to PAMPs involving ModSP and Grass each have a significant *in vivo* role in regulating Toll responses. Whereas Toll signaling remains readily observable in mutants lacking either *psh* or *grass* function, knocking out both systems eliminates Toll pathway activity (El Chamy et al., 2008).

Initiation of innate immune Toll signaling is subject to negative regulation at several points. In particular, serpins (serine protease inhibitors) inhibit the activity of a number of key immune response proteases. For example, the serpin Necrotic (nec or Spn43Ac) helps maintain Persephone in an inactive state in the absence of infection. This inhibitory control by Necrotic is essential to normal immune homeostasis: Toll activation occurs in response to either loss of Necrotic function (Levashina et al., 1999) or overexpression of Persephone (Ligoxygakis et al., 2002b).

Serpin activity is also important in regulating the response to fungal PAMPs. In particular, the serpin Spn1 (Spn42Dd) has a significant antagonistic role in the response to fungal cell wall components. Inactivation of the Spn1 increases induction of Toll regulated response loci, whereas overexpression of Spn1 inhibits signaling in response to fungal infection (Fullaondo et al., 2011). Epistasis analysis places Spn1 upstream of Grass and downstream of GNBP3, making ModSP a likely target (see Fig. 2).

4. Drosophila humoral immunity

A hallmark of insect innate immunity is the rapid and massive induction of antimicrobial peptide (AMP) genes (Steiner et al.,

1981). Released into insect hemolymph, which acts as both circulatory and interstitial fluid, AMPs kill microbes or block their growth by disrupting membrane integrity. In *Drosophila*, there are roughly 20 AMP loci; most encode small (<100 residues), secreted, cationic peptides. Induction is remarkably robust, with the concentration of the anti-fungal AMP Drosomycin reaching 100 μM in the hemolymph (Fehlbaum et al., 1994).

Two *Drosophila* recognition and response pathways trigger induction of AMP genes and other immune loci upon systemic infection. One is the Toll pathway, the other is the Imd (immune deficiency) pathway (Lemaitre et al., 1995a; Rutschmann et al., 2000; Silverman et al., 2000). The Imd pathway is specifically activated by bacterial peptidoglycan that contains *meso*-diaminopimelic acid (DAP) at the third position of the stem peptide (Choe et al., 2002; Kaneko and Silverman, 2005; Kaneko et al., 2006). This DAP-type PGN structure is characteristic of all Gram-negative bacteria and a few Gram-positive genera, including *Bacillus* and *Clostridium*. The Imd pathway thus detects a group of bacteria complementary to those detected by the Toll pathway.

In composition and in mechanism, the Imd pathway bears similarity to the mammalian TNFR1 pathway. Upon binding DAP-type PGN, the transmembrane receptor PGRP-LC alone, or in coordination with the related protein PGRP-LE, recruits Imd, a homolog of the mammalian receptor interacting protein (RIP) (Choe et al., 2005; Ferrandon et al., 2007; Georgel et al., 2001). Activated Imd then signals through a branched pathway that includes orthologs of the mammalian TAK protein (a MAP kinase kinase kinase) and IKK (IκB kinase) proteins (Lu et al., 2001; Silverman et al., 2003). The target of signaling is the transcription factor Relish, which, like mammalian p105, contains an N-terminal Rel domain and a C-terminal IκB-like autoinhibitory domain. Acting in part via a caspase that cleaves a target site between these domains, the pathway effects Relish activation (Erturk-Hasdemir et al., 2009; Meinander et al., 2012; Stoven et al., 2003).

Flies deficient for both the Imd and Toll pathways fail to induce any of the known antimicrobial peptides and succumb readily to infection (Tzou et al., 2002). Moreover, activation of either pathway by, for example, overexpressing a pathway component, is sufficient to trigger AMP expression in the absence of infection (Georgel et al., 2001; Tauszig-Delamasure et al., 2002). Toll and Imd are thus necessary and sufficient for the major humoral response of *Drosophila*.

In response to infection, the Toll and Imd pathways each direct expression of a set of immune response loci. Some loci are pathway specific, whereas others can be induced by either the Toll pathway or the Imd pathway (De Gregorio et al., 2001, 2002; Hedengren-Olcott et al., 2004; Imler et al., 2004; Irving et al., 2001; Lemaitre et al., 1997). Are the responses matched to the distinct inducers? Yes, at least in some cases. For example, fungi activate Toll, but not Imd, signaling and it is Toll that directs expression of the AMPs with the best-characterized antifungal activity *in vitro* – Metchnikowin and Drosomycin (Fehlbaum et al., 1994; Levashina et al., 1995).

Do Drosomycin and Metchnikowin in fact have a major role in the fly's antifungal defense? Answering such a question about the *in vivo* function of particular AMP loci is not trivial. Genetic screens focused on immunity have not yielded loss-of-function mutations in individual AMP genes. The failure of AMP loci to be identified in this way likely reflects a combination of the screening strategies employed, the small target size of individual AMP genes, and the expected functional redundancy among sets of related AMPs. There has been, however, one elegant and informative study that circumvented this problem. In it, individual AMP loci were heterologously expressed in flies defective for both Toll and Imd signaling. By itself, this immunodeficient background rendered lethal an infection by many bacteria and fungi to which wild-type flies are resistant (Tzou et al., 2002). In this background, expression of Drosomycin alone restored wild-type resistance to the fungus *Neurospora crassa*, demonstrating that Drosomycin contributes significantly to anti-fungal defenses induced by the Toll pathway.

How do Toll and Imd direct expression of distinct but overlapping gene sets? Each pathway regulates a Rel family transcription factor: Dif (or Dorsal) alone is sufficient to mediate Toll responses and Relish alone is sufficient to mediate Imd responses. Each binds to sequence motifs in the DNA termed κB sites. It turns out that a regulatory code based on site number and sequence governs regulation of AMP genes and other innate immune loci by Toll and Imd (Busse et al., 2007). Loci regulated only by the Toll pathway have a single κB site characterized by a GGGAA consensus half-site. In contrast, loci under only Imd control have multiple κB sites with a consensus half-site sequence of GGGGA. Bioinformatic analyses, *in vitro* binding studies, and experiments with transfected cultured cells confirm the validity of this code (Busse et al., 2007).

Some innate immune loci are regulated by both Toll and Imd signaling (De Gregorio et al., 2001, 2002). How is their expression regulated? There are two competing hypotheses. One is that Dif (or Dorsal) induced by Toll heterodimerizes with Relish induced by Imd, as observed when expression of both factors is driven in the same cells (Han and Ip, 1999; Tanji et al., 2007, 2010). The resulting heterodimer would be analogous in structure to NF-κB, which is comprised of p65 and p50, and would have a DNA binding specificity distinct from either subunit. Dif and Dorsal closely resemble p65 in structure and sequence, and the cleaved form of Relish is comparable to the p50 cleavage product of p105. Furthermore, a tethered chimera of Dif and Relish is active *in vivo* (Tanji et al., 2010). It is likely, however, that the individual domains of the chimera can form intermolecular homodimers, which could be responsible for the observed activity. Moreover, heterodimers of Dif and Relish appear to be scarce in fly cells (Han and Ip, 1999), unlike the near ubiquity of p50/p65 heterodimers in mammalian cells expressing both proteins.

The alternative hypothesis for co-regulation of loci by Toll and Imd is the presence of κB sites that separately recruit Dif and Relish to the same promoter proximal region (Busse et al., 2007). This model, illustrated in Fig. 3, is consistent with site analysis of loci subject to dual regulation. Assuming κB sites act cooperatively, this model, like the alternative, can readily explain the synergistic effect on some loci of activating both pathways (Busse et al., 2007; Tanji et al., 2010).

5. A non-conventional pathway for Toll-related receptors

The Toll gene family has multiple members in the majority of animal species. To date, the sea urchin (*Strongylocentrotus purpuratus*), which has 253 Toll loci, represents the zenith for documented duplication and divergence (Buckley and Rast, 2012; Buckley and Smith, 2007). Humans have ten TLRs, each functioning as a PRR. TLR4, for example, recognizes LPS, whereas TLR-3, -7, -8, and -9 recognize viral nucleic acids (Kawai and Akira, 2011; Poltorak et al., 1998).

Flies encode eight Toll-related receptors (Toll-2–9) in addition to Toll itself. Furthermore flies have five genes encoding homologs of Spätzle. It might therefore seem reasonable to hypothesize that distinct pairs of Spz and Toll related proteins mediate particular immune responses to particular pathogens. Yet until recently there had been little evidence to support such a hypothesis. A mutation mapping to Toll-2 (18-wheeler) impaired immune induced AMP expression, but turned out to perturb development of immune tissues and perhaps affect a neighboring gene rather than Toll-2 (Ligoxygakis et al., 2002a; Williams et al., 1997). The Toll-related receptor Toll-9 was suggested to activate a constitutive innate

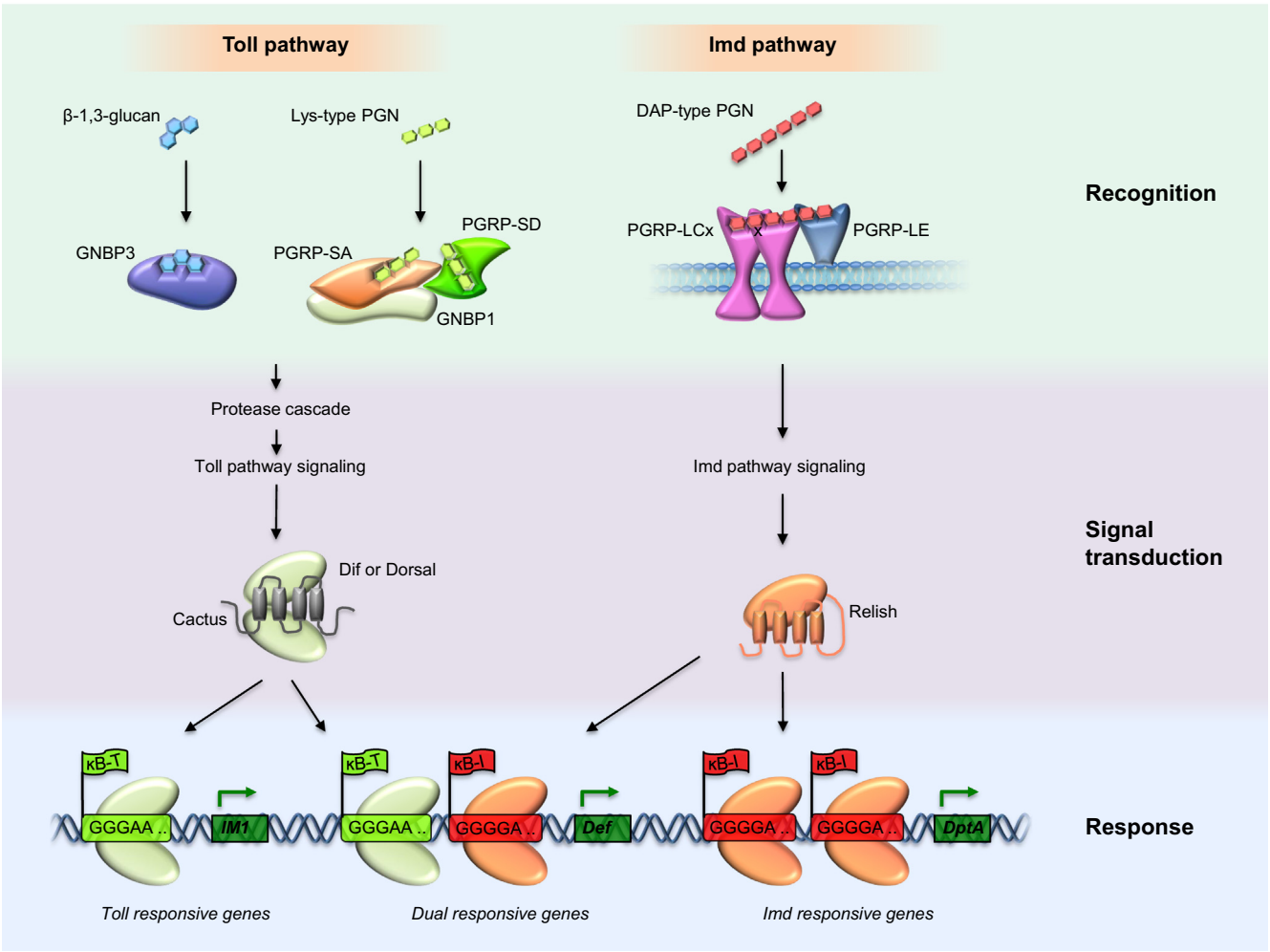

Fig. 3. Specificity and synergy in Toll and Imd signaling. The Toll and Imd pathways recognize distinct PAMPs and generate distinct responses, with either separate or coordinate regulation of transcriptional outputs. In the case of fungi and most Gram-positive bacteria, cell wall components (β-1,3,-glucan from fungi; Lys-type peptidoglycan from bacteria) are recognized by extracellular pathogen recognition receptors and the signal is transduced through protease cascades to activate Toll pathway signaling. A pure Toll response involves binding of a homodimer of the transcription factor Dif or Dorsal, typically at a single Toll-specific κB site (κB-T) upstream of Toll-responsive genes. In the case of Gram-negative bacteria, and select Gram-positive species, polymeric DAP-type peptidoglycan (PGN) is recognized by a dimer of PGRP-LCx and an extracellular version of PGRP-LE (containing only the PGRP domain) to activate Imd signaling. A pure Imd response involves two or more homodimers of the transcription factor Relish binding at neighboring Imd-specific κB sites (κB-I) upstream of Imd-responsive genes. In the event that both pathways are stimulated, Toll- and Imd-regulated Rel proteins can cooperatively regulate a third set of genes. The promoters of such dual-responsive genes contain neighboring Toll-specific and Imd-specific κB sites, where homodimers of Dif or Dorsal and of Relish, respectively, can bind to effect transcription. IM1: immune-induced molecule 1. Def: defensin. DptA: diptericin A.

immune defense (Ooi et al., 2002), but loss of Toll-9 function does not lead to an antibacterial response defect (Narbonne-Reveau et al., 2011). Furthermore, experiments reveal an effect on immune responses of inactivating Toll, but little effect of inactivating nearly all of the Toll-related receptors (Bilak et al., 2003; Luo et al., 2001; Tauszig et al., 2000; Yagi et al., 2010).

One insightful way to address the function of the fly Toll-related receptors comes from considering downstream adaptors. Mammals have four adaptors required for signaling by one or more TLRs: MyD88, TRIF, TRAM, and Mal (also known as TIRAP). Of the four, only MyD88 is found in flies. Significantly, an inactivating mutation in MyD88 has the same phenotypes in a range of immune function assays as a loss-of-function mutation in Toll. Mutations in Tube and Pelle similarly resemble Toll mutations. It thus appears that the immune function of the conventional Toll pathway is provided exclusively by Toll, and not the fly Toll-related receptors.

Could fly Toll-related receptors signal through a non-conventional pathway? A potential breakthrough in this regard came from a study on the immune response in the fly trachea. It had been shown that the Imd pathway controls AMP production in tracheae, as in many other epithelial tissues (Ferrandon et al., 1998; Wagner et al., 2008). However, recent experiments provide evidence that one of the fly Toll-related receptors, Toll-8, negatively regulates this function of Imd (Akhouayri et al., 2011). Inactivation of Toll-8 significantly increases the strength of the immune response in trachea and, in particular, the transcription of AMP loci induced by the Imd pathway. Furthermore, providing Toll-8 activity constitutively inhibits Imd-mediated AMP expression.

Although Toll-8 was found to inhibit Imd signaling in trachea, mutating MyD88 had no effect. Furthermore, neither Tube nor Pelle is expressed in this tissue (Wagner et al., 2008). How then does Toll-8 carries out its inhibitory role? The answer apparently lies in the fly homolog of SARM, the broadly conserved Sterile-Alpha and Armadillo Motif protein (Kenny and O'Neill, 2008; Mink et al., 2001). In mammals, SARM does not promote TLR signaling or other immune responses, but instead exerts an inhibitory effect on TLR3 and TLR4 pathways (Peng et al., 2010; Yuan et al., 2010). When SARM (Ect4) was inactivated in *Drosophila* trachea, the effect was the same as blocking Toll-8 function (Akhouayri et al., 2011).

Filling out the story further, inactivating mutations in *spz2* (the fly neurotrophin 1 gene) behave identically to mutations in Toll-8 or SARM, i.e., they enhance the strength of the Imd response (Akhouayri et al., 2011). Furthermore, constitutive overexpression of Spz2 or of SARM suppressed Imd-mediated AMP expression. Thus, for the first time there is evidence for the function of a fly Toll-related receptor in a non-conventional signaling pathway.

How might the Spz2/Toll-8/SARM pathway function? Although the exact activity of SARM in the mammalian TLR3 and TLR4 pathways is not known, experiments demonstrate that it binds to the TLR adaptor TRIF and prevents signaling from TRIF to RIP (Carty et al., 2006). Given that PGRP-LC, like TRIF, contains a RHIM (RIP homotypic interaction motif) domain and that Imd is a RIP homolog, it seems reasonable to speculate that SARM similarly interferes with the recruitment and activation of Imd by PGRP-LC. This interaction might help maintain the Imd pathway in an inactive state in the absence of infection or provide a route for down-regulation after an initial induction.

Is the non-conventional pathway mediated by Toll-8 dedicated to immunity? Probably not. Toll-8 was previously implicated in neuron-specific glycosylation and neural patterning during embryogenesis (Ayyar et al., 2007; Seppo et al., 2003). Furthermore, *Drosophila* SARM was identified in a screen for mutations that inhibit Wallerian degeneration – the disintegration and death of an axon that has been severed from the neuron cell body (Osterloh et al., 2012). There is also good evidence that SARM functions in neuronal tissue in other organisms, including *Caenorhabditis elegans*, which lacks canonical Toll signaling in immunity (Chuang and Bargmann, 2005; Couillault et al., 2004).

What of the remaining *Drosphila* Toll-related receptors? At least one, Toll-7, appears to function in antiviral defenses. Major antiviral defenses in flies include the Dicer-2 mediated response to dsRNA (RNAi) and activation of the JAK-STAT pathway (Deddouche et al., 2008; Dostert et al., 2005; Wang et al., 2010). One of the additional antiviral defenses involves autophagy in response to infection by particular viruses, such as vesicular stomatitis virus (VSV) (Shelly et al., 2009). A recent study indicates that Toll-7 activates antiviral autophagy and that inactivating Toll-7 in VSV-infected flies increases viral RNA production (Nakamoto et al., 2012). Whether SARM and a Spz family member also participate has not yet been established.

6. Conclusions

Work in the last few years has clarified a number of questions regarding the composition and organization of the canonical Toll pathway. There remain, however, underexplored areas. Toll signaling in embryos and larvae contributes to hematopoiesis and blood cell survival, as well as to patterning at the neuromuscular junction, but the nature of these contributions is as yet ill defined (Halfon and Keshishian, 1998; Heckscher et al., 2007; Matova and Anderson, 2010; Qiu et al., 1998). Furthermore, RNAseq data reveal robust Dorsal expression in adult males, suggesting that Dorsal's function extends beyond maternally directed embryonic patterning and larval innate immunity.

Why evolution resulted in PAMP binding by mammalian TLRs but an endogenous ligand for *Drosophila* Toll remains a subject of lively debate. One idea is that the presence of protease cascades upstream of fly Toll might indicate a past fusion of the Toll pathway with a more ancient defense cascade, such as that triggering melanization (Cerenius et al., 2010). It is worth noting in this regard that recent studies of wound healing in flies have demonstrated an interrelationship of the wound response pathways both with protease cascades and with Toll signaling (Juarez et al., 2011; Markus et al., 2005).

Work on the Toll-related receptors and their alternative signaling in flies is in its early stages. Is Spz2 a Toll-8 ligand? It seems likely, although this pairing was not anticipated by structural considerations of the Toll-related receptors (Gangloff et al., 2013). Does SARM act in conjunction with other fly Toll-related receptors? The idea is certainly appealing and is consistent with the fact that *Drosophila* SARM, unlike Toll, is an essential gene. Are there additional components to the non-conventional pathway? One expects so, but only time, and more experiments, will tell.

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