The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections

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Abstract | A hallmark of the potent, multifaceted antimicrobial defence of *Drosophila melanogaster* is the challenge-induced synthesis of several families of antimicrobial peptides by cells in the fat body. The basic mechanisms of recognition of various types of microbial infections by the adult fly are now understood, often in great detail. We have further gained valuable insight into the infection-induced gene reprogramming by nuclear factor-κB (NF-κB) family members under the dependence of complex intracellular signalling cascades. The striking parallels between the adult fly response and mammalian innate immune defences described below point to a common ancestry and validate the relevance of the fly defence as a paradigm for innate immunity.

Haemolymph
Insects have an open circulatory system. The haemolymph is the blood-like fluid that bathes tissues and is circulated throughout the body cavity by the dorsal vessel, a functional equivalent of the heart.

Melanization
The deposition of melanin at the site of injury as a result of the activation of a biochemical cascade involving a key enzyme, phenol oxidase, which is activated in response to septic injury. This activation releases toxic reactive oxygen species that may attack invading microbes. Although this mechanism is highly conserved in invertebrates, a primary role of melanization in host defence remains to be firmly established.

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It has been known for more than a century that flies are strongly resistant to microbial infections. Defence reactions in insects include the activation of proteolytic cascades in the haemolymph that lead to melanization of the invading microbe at the site of injury, cellular responses, namely phagocytosis or encapsulation of foreign material, and a potent humoral systemic response. The analysis of the humoral systemic response of insect host defence gained momentum in the early 1980s with the isolation from bacteria-challenged pupae of the moth *Hyalophora cecropia* of two groups of inducible antimicrobial peptides (AMPs) that were shown to be effectors of this response in moths[1]. Numerous distinct AMPs were subsequently identified in many insect species, including *Drosophila melanogaster*. In this species, it was established that an experimental microbial challenge induces the synthesis of seven families of peptides or polypeptides with distinct activities directed against fungi, Gram-positive bacteria or Gram-negative bacteria by cells in the fat body, which is analogous to the mammalian liver[2] (see Supplementary information S1 (table)).

The identification of these inducible molecules and the cloning of their corresponding genes in the early 1990s were rapidly followed by analysis of the challenge-induced control of their expression. Benefiting from the powerful genetics that can be carried out in *D. melanogaster*, these studies established by the mid-to-late 1990s that the expression of the AMP genes depends on two *D. melanogaster* members of the nuclear factor-κB (NF-κB) family of inducible transactivators: DIF (dorsal-related immunity factor) and Relish. DIF is mainly activated in response to fungal and Gram-positive bacterial infection, whereas Relish is preferentially activated by Gram-negative bacterial infection in adult flies. Of great interest was the discovery that the activation of DIF and Relish in response to fungal and bacterial infection occurred through two distinct signalling cascades, which are now known as the Toll and immune deficiency (IMD) pathways, respectively[3]. The Toll signalling pathway involves several factors that were initially discovered in the control of dorsoventral patterning in the embryo[4] and it has some parallels to the mammalian signalling cascades downstream of the interleukin-1 receptor (IL-1R) and the Toll-like receptors (TLRs). By contrast, the IMD pathway is similar to the tumour-necrosis factor-receptor (TNFR) pathway in mammals[5].

By the end of the 1990s and early into the twenty-first century the *D. melanogaster* proteins that sense invading microbes were functionally characterized. Remarkably, these recognition proteins seem to be derived from phylogenetically ancient amidases and glucanases that were first discovered and characterized in silkworms[6]. Consequently, the main microbial inducers that have been identified to date are various forms of peptidoglycans and glucans[7–9]. These recognition proteins, as well as other *D. melanogaster* innate immunity receptors involved in signalling and phagocytosis, are shown in FIG. 1.

Fifteen years after the initial studies into the molecular and genetic mechanisms that underlie the potent inducible antimicrobial defence of *D. melanogaster*, a general picture of this response has evolved. We review this systemic immune response here with emphasis on the control of gene reprogramming following an immune challenge, as gained from the analysis of mutant phenotypes in adult *D. melanogaster* (BOX 1).

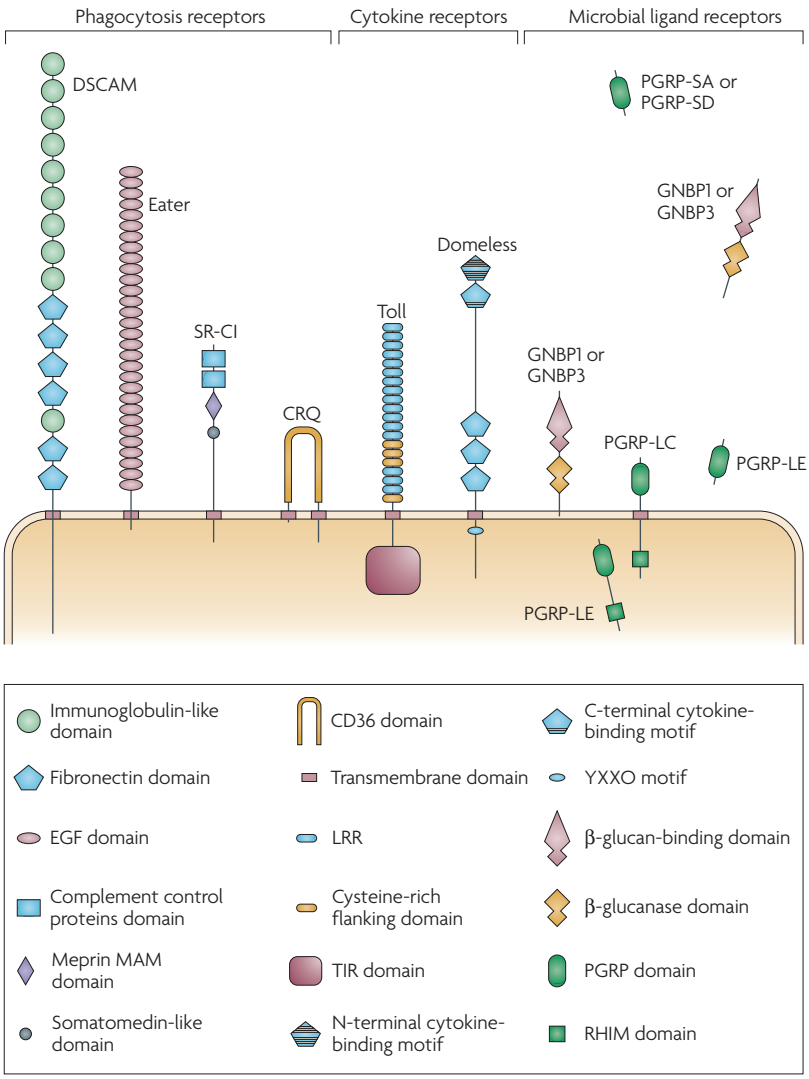


Figure 1 | **Innate immune receptors in *Drosophila melanogaster*.** Different types of receptors participate in immune defence in *D. melanogaster*. Transmembrane receptors expressed by haemocytes are involved in phagocytosis. Note that the roles of down syndrome cell-adhesion molecule (DSCAM), scavenger receptor CI (SR-CI) and Croquemort (CRQ) have only been tested in cell lines, and their contribution to host defence *in vivo* remains to be established. Other receptors with a role in immune defence include cytokine receptors, namely Toll, which shares the cytoplasmic TIR (Toll/interleukin-1 receptor (IL-1R)) domain with mammalian Toll-like receptors (TLRs), and Domeless, which has similarities to the gp130 subunit of the IL-6R and activates the JAK–STAT (Janus kinase–signal transducer and activator of transcription) pathway. The receptors that trigger signalling in response to sensing microbial ligands belong to two structural families: the peptidoglycan-recognition proteins (PGRPs) and the Gram-negative binding proteins (GNBPs). They exist as transmembrane receptors (for example PGRP-LC), but can also be associated to the plasma membrane by a glycosylphosphatidylinositol anchor (for example GNBPs), or be secreted and function as soluble receptors (for example PGRP-SA, PGRP-SD, PGRP-LE, GNBP1 and GNBP3). One member of the PGRP family, PGRP-LE can also function as an intracellular receptor for peptidoglycan in some cell types. EGF, epidermal growth factor; LRR, leucine-rich repeat; RHIM, RIP (receptor-interacting protein) homotypic interaction motif.

Sensing infection

Recognition of DAP-type Gram-negative bacteria. The *D. melanogaster* immune system can discriminate between distinct classes of microorganisms[10]. Thus, the IMD pathway is preferentially induced by Gram-negative bacteria (and some Gram-positive bacilli) and this pathway controls the host defence against these infections. Although lipopolysaccharides (LPS) form the outer cell layer of Gram-negative bacteria, they do not activate the IMD pathway[7,8]. Beneath the external LPS coat and outer membrane of Gram-negative bacteria, peptidoglycan (PGN) forms an inner layer of polymeric glycan chains that are crosslinked by peptidic stems (see Supplementary information S2 (figure)). The third amino acid of the PGN peptidic stems is a meso-diaminopimelic acid (DAP) residue in Gram-negative bacteria (or an amidated-DAP in Gram-positive bacilli). By contrast, many medically important Gram-positive bacteria have a lysine (Lys) residue in this position. *D. melanogaster* can discriminate between these two types of PGN (DAP-type PGNs or Lys-type PGNs) using PGN-recognition proteins (PGRPs).

The PGRP family comprises 13 members, of which at least one (PGRP-LC) can be further diversified by alternative splicing[11]. Family members share a common PGRP domain, which is evolutionarily related to the bacteriophage type II amidases, and some members have retained this enzymatic activity (these are referred to as catalytic PGRPs)[12,13]. By contrast, other PGRPs have lost crucial amino-acid residues that are essential for catalysis and they serve as microbial sensors (these are referred to as recognition PGRPs)[14].

PGRP-LC and PGRP-LE, two non-catalytic members of the PGRP family, mediate the detection of Gram-negative bacteria and activation of the IMD pathway[15–21]. PGRP-LC is the main transmembrane (type II) receptor of the IMD pathway, whereas PGRP-LE is a cleaved, secreted PGN sensor in the haemolymph. Interestingly, uncleaved PGRP-LE can function as an intracellular sensor in Malpighian tubules[15–17,19,20] (FIG. 1). PGRP-LC and PGRP-LE bind directly to and are activated by DAP-type PGNs or shorter PGN end fragments such as tracheal cytotoxin (TCT)[7,8,18,20,22–27].

The gene encoding the transmembrane PGN sensor PGRP-LC produces three distinct splice isoforms, a, x and y, that each code for a distinct extracellular PGRP domain[11]. Cell-culture studies indicate that PGRP-LCx homodimers sense polymeric DAP-type PGN and that PGRP-LCx–PGRP-LCa heterodimers are required for the detection of TCT (TABLE 1). The X-ray structure of the PGRP domains of free or TCT-bound PGRP-LC or PGRP-LE has yielded a unique insight into the mechanisms of discrimination between DAP-type and Lys-type PGNs[26,27] (FIG. 2Aa). Two important features account for the preferential binding of TCT to these receptors. One is the formation of a strong electrostatic bond between two negatively charged groups of DAP and an arginine

Peptidoglycan-recognition proteins
(PGRPs). This family is characterized by the presence of one or several PGRP domains that present similarities to bacteriophage amidases. This family has been conserved throughout evolution. Catalytic and non-catalytic PGRPs are also found in vertebrates in which they act as amidases or antimicrobial peptides.

Malpighian tubules
The excretory and osmoregulatory organs of insects that open near the junction of the midgut and hindgut.

Box 1 | **Experimental systems**

The advantage of *Drosophila melanogaster* as an experimental model is that immunity can be studied at the level of the whole organism by monitoring the resistance of mutant flies to infections (20–50 flies per genotype). A phenotype of sensitivity to infection usually correlates with an increased microbial titre in infected flies and a decrease in the induction of either the Toll or immune deficiency (IMD) pathways. The induction of these pathways is usually monitored by measuring the transcript levels of antimicrobial peptide (AMP) genes (*Diptericin* for the IMD pathway; *Drosomycin* for the Toll pathway; and other AMPs, such as Cecropins, Attacins or Defensin, are expressed within a few hours of an immune challenge under the control of both pathways).

Mutants in which these pathways were affected had either been generated in screens for developmental genes (Toll pathway), existed in the background of other mutations (*imd* itself) or were found by forward or reverse genetics. To achieve this, besides transposon insertions, a novel technique is available that relies on RNA interference triggered by expressing a transgene containing a hairpin construct of the targeted gene in a tissue-specific and/or temporally controlled manner. A genome-wide library containing more than 20,000 transgenic lines is now available to the *D. melanogaster* community[116].

In this Review, we focus on the systemic immune response in adult flies. However, during their development, the flies first go through distinct larval stages and live within a highly septic environment, such as decaying fruits, to which adult flies are less exposed. Although a systemic immune response exists in larvae as well, there are features specific to this developmental stage that cannot be extended to adults. These include a fat body that is distinct from that of adults, hormonal control of AMP gene expression (larvae must be accurately staged to avoid artefacts), and haemocytes that are more numerous than in adults. Therefore, the cellular immune response is likely to have a more prevalent role in larvae[117].

Genome-wide RNA interference screens have been performed in cultured S2 cells[118,119]. However, the genes identified in such studies need to be validated *in vivo*.

residue present in the PGRPs that bind DAP-type PGN (FIG. 2Ac). The second feature relies on the existence of two distinct binding sites on the PGRP domain. One site binds directly to TCT through an L-shaped binding groove. The second site binds to TCT that is already bound to the binding groove of another PGRP domain (FIG. 2Ab). The formation of PGRP homodimers — or possibly heterodimers — significantly contributes to the high affinity of PGRP complexes for TCT[26].

The relationship between PGRP-LC and PGRP-LE is complex. Genetically, PGRP-LC is strictly required for resistance to several species of Gram-negative bacteria (for example, *Enterobacter cloacae* or *Erwinia carotovora*)[15,19] and is partially required for the activation of the IMD pathway by Gram-negative bacterial challenge, as shown by the decreased, but not abolished, levels of expression of antibacterial peptide genes in the absence of PGRP-LC[15–17]. By contrast, PGRP-LE mutants are not susceptible to most Gram-negative bacterial infections[19]. Interestingly, only flies that are mutant for both PGRP-LC and PGRP-LE are sensitive to *Escherichia coli* infection and fail to express *Diptericin*, a common read-out of IMD-pathway activation, after injection of TCT[19,20]. Therefore, PGRP-LC and PGRP-LE can synergize under some conditions in the adult fly to activate the IMD pathway. Overexpression of *PGRP-LC* or *PGRP-LE* induces the IMD pathway in the absence of infection[15,18,19]. The effect obtained by overexpressing *PGRP-LE* in flies partially requires *PGRP-LC*[19], in keeping with a similar observation in a cell culture system[20]. Taken together, these experiments suggest that secreted PGRP-LE activates the IMD pathway through PGRP-LC, possibly by forming PGRP-LC–PGRP-LE heterodimers.

Table 1 | **Microbial elicitors and their cognate pattern recognition receptors**

Ligand	Pathogen	*Drosophila melanogaster*
Polymeric DAP-type PGN	Gram-negative bacteria	PGRP-LCx
TCT	Gram-positive bacilli	PGRP-LCx–PGRP-LCa; PGRP-LE–PGRP-LC?
Lys-type PGN	*Micrococcus luteus*; *Enterococcus faecalis*	PGRP-SA–GNBP1
	Streptococcus pyogenes; *Staphylococcus saprophyticus*; *Staphylococcus aureus*	PGRP-SA–GNBP1; PGRP-SD
Polymeric β-(1,3)-glucans	*Candida spp.*; *M. anisopliae*	GNBP3 (PGRP-LC?)

DAP, diaminopimelic acid; GNBP, Gram-negative binding protein; PGN, peptidoglycan; PGRP, PGN-recognition protein; TCT, tracheal cytotoxin.

Analysis of the current literature leads to the proposal of the following model for the sensing of Gram-negative bacteria by PGRPs in adult flies. During septic (or intestinal) infection, bacteria release short PGN fragments, such as TCT, as a result of cell-wall remodelling during growth and division. These PGN fragments are detected by PGRP-LCx–PGRP-LCa[23,24], and possibly PGRP-LCx–PGRP-LE, heterodimers[19,20], and these in turn activate the IMD pathway. As a consequence of this early activation, effectors of the humoral immune response (such as AMPs and lysozymes) attack the invading bacteria, and this leads to the release of large fragments of polymeric DAP-type PGN that were initially hidden under the LPS outer coat. These fragments can then be sensed directly by an array of membrane-bound PGRP-LCx receptors (FIG. 2B)[26].

The intensity of the stimulation of the IMD pathway can be modulated by the catalytic PGRPs (PGRP-SC1, PGRP-SB1 and PGRP-LB), which cleave the amide bond between the muramic acid of the glycan chain and the DAP-containing peptide stem of PGN[12,13,28]. PGN that has been digested by PGRP-SC1 or PGRP-LB is barely immunostimulatory and these catalytic PGRPs thereby function as scavengers[12,28]. Indeed, flies deficient for either of these PGRPs have a more intense and longer-lived activation of the IMD pathway following bacterial infection[28,29]. PGRP-SC1, PGRP-SB1, and PGRP-LB

can all cleave DAP-type PGN, but PGRP-LB is the only PGRP that can cleave TCT[13,28]. An additional difference is that the closely related PGRP-SC1 and PGRP-SC2 are constitutively expressed, mostly in the gut, whereas PGRP-LB and PGRP-SB1 are induced by the IMD pathway[11,28,30]. We propose that early activation of the IMD pathway is mostly mediated by TCT, which is resistant to modulation by all the catalytic PGRPs that are present

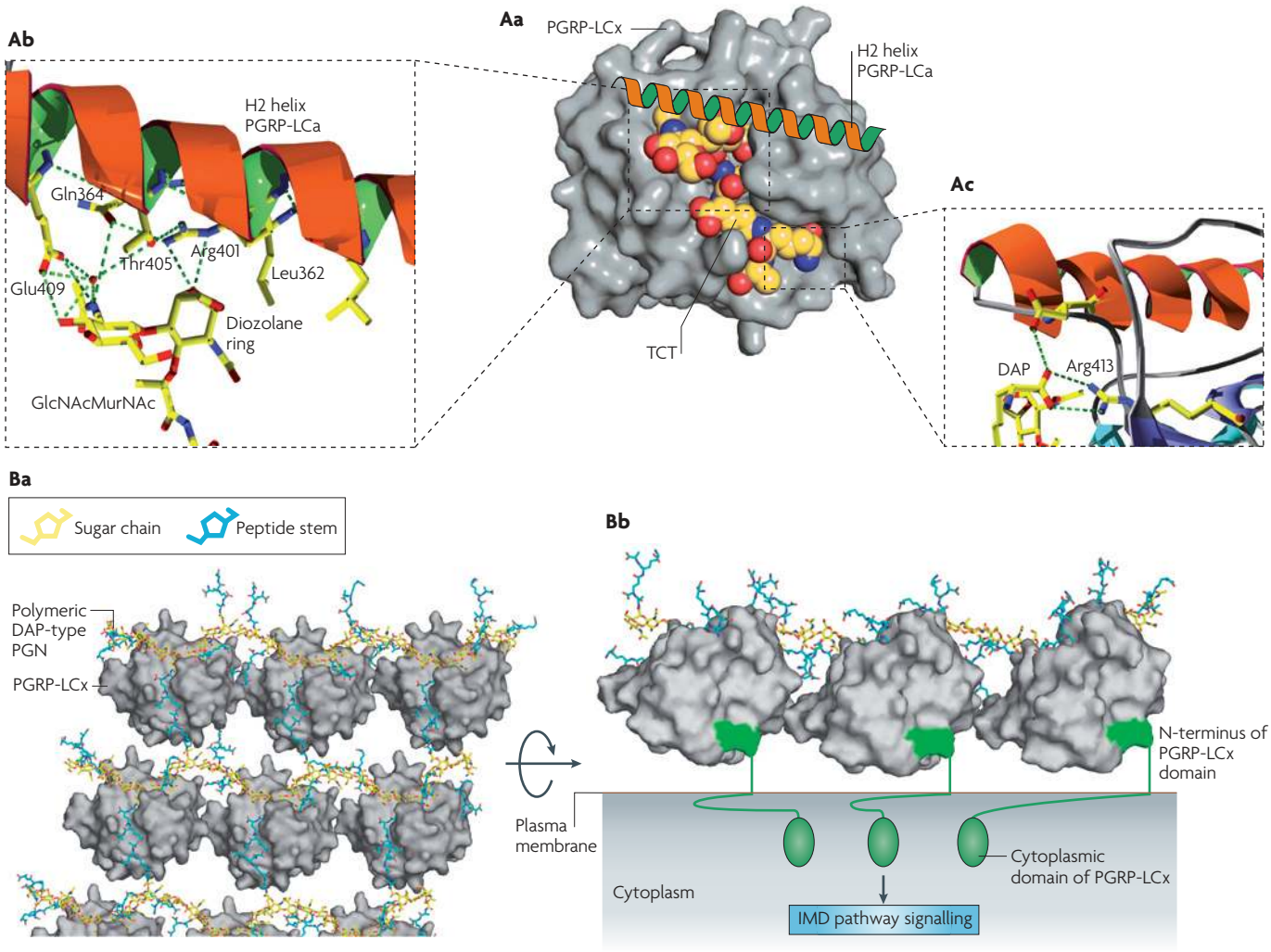


Figure 2 | **Molecular basis for the differential recognition of microbial structures. Aa** | The tracheal cytotoxin (TCT; shown in yellow) and peptidoglycan-recognition protein-LCx (PGRP-LCx; shown in grey) complex, as viewed from the extracellular space towards the cell membrane. The complex is represented in a space-filling model to illustrate the close fit between TCT and the L-shaped binding groove of the PGRP-LCx ectodomain. The position of the H2 helix from the PGRP-LCa ectodomain, which heterodimerizes with TCT-bound PGRP-LCx, is indicated. Modified with permission from REF. 27 © (2006) American Association for the Advancement of Science. Note that PGRP-LCa cannot bind TCT on its own, as the TCT-binding groove has not been conserved in this domain[23,24]. Therefore, PGRP-LCa can only bind to TCT that is presented by PGRP-LCx through its dimerization domain. **Ab** | The interactions between the disaccharidic moiety of TCT (represented in yellow sticks) and the H2 helix of PGRP-LCa . The residues that form hydrogen bonds (green dotted lines) with TCT are shown. The (1,6) anhydrobond present in the muramic acid of TCT (MurNAc(anh)) or at the end of peptidoglycan (PGN) chains is an important determinant of this interaction. For simplicity, PGRP-LCx, located beneath TCT, has not been drawn. PGRP-LCx forms several hydrogen bonds with MurNAc(anh) and also makes hydrophobic contacts with residues of the PGRP-LCa H2 helix. **Ac** | The diaminopimelic acid (DAP) residue in the peptidic stem of TCT is characterized by a carboxyl group that forms a bidentate salt bridge with the guanidinium of Arg413 of PGRP-LCx. A functionally important Arg residue is present at the same position in PGRP-LE[26], but not in PGRP-SA in which a non-charged Thr amino acid is found at this position, consistent with the preferred binding of PGRP-SA to Lys-type PGN. **B** | Model of the interaction between polymeric DAP-type PGN from *Escherichia coli* and an array of PGRP-LCx receptors. View from the extracellular side toward the cell membrane (**a**). Transverse view through the membrane, illustrating how the binding of the PGRP domains to TCT induces a close proximity of the intracellular domains of the receptor that presumably initiates immune deficiency (IMD) pathway signalling (**b**). Modified with permission from REF. 26 © (2006) American Society for Biochemistry and Molecular Biology.

before infection. PGRP-SB1 and PGRP-LB, which need to be induced, would act later in the immune response, both on TCT and on polymeric DAP-type PGN, to downregulate the response in a putative autocrine feedback loop.

It is striking that PGRP receptors can bind the PGN of Gram-negative bacteria, as PGN is not directly accessible for binding. Two categories of models can be proposed for the immune system to circumvent this apparent difficulty. In the first category, bacteria could be attacked by haemocytes and/or AMPs, which might basally be expressed at low levels, and this would result in the release of PGN fragments in the haemolymph. Phagocytosis of bacteria by haemocytes could also lead to indirect activation of the IMD pathway by an unknown signal. In larvae (see BOX 1), the analysis of the *psidin* mutant phenotype supports this first model category, as *psidin* is required in haemocytes for the induction of the AMP gene *Defensin* in the fat body and is also required for the degradation of ingested bacteria[31]. However, it is puzzling that the expression of only one of seven established AMP genes is consistently affected in *psidin* mutant larvae. The second category of models postulates that bacteria could release short PGN fragments during proliferation and growth as a result of cell-wall remodelling. The finding that TCT fed to flies reaches the haemolymphatic compartment and triggers the systemic induction of the IMD pathway in cells in the fat body is in agreement with such a model[28]. Furthermore, it has recently been shown in an oral infection model that ingested *Serratia marcescens* bacteria fail to induce the IMD pathway despite their presence in significant numbers in the haemocoele after passage through the intestinal barrier[124]. Interestingly, a strong systemic response was only observed when phagocytosis was blocked, an experimental situation that correlates with a strong proliferation of these bacteria. Therefore, the innate immune system may sense proliferation of bacteria rather than their presence, a hypothesis that could explain why the potential endogenous bacterial flora fails to induce a systemic immune response.

Recognition of Lys-type Gram-positive bacteria. Lys-type Gram-positive bacteria are also sensed by members of the PGRP family, namely PGRP-SA and PGRP-SD[32,33], and by GNBP1, a member of the Gram-negative binding proteins (GNBPs; also known as β-glucan recognition proteins (βGRP)) family[34,35,36] (FIG. 1). Sensing of Gram-positive bacteria (and fungi) results in the activation of proteolytic cascades that culminate in cleavage of the cytokine Spätzle, which is the ligand for the transmembrane receptor Toll.

PGRP-SA binds preferentially to Lys-type PGN[37,38], in keeping with the absence of the arginine residue that binds specifically to the carboxyl group of DAP-type PGN. GNBP1 binds to a more restricted range of Lys-type PGN than does PGRP-SA[38]. GNBP1 functions together with PGRP-SA in sensing some Gram-positive bacterial strains[34] (TABLE 1). Although the glucanase domain of GNBP1 is predicted to be catalytically inert, GNBP1 can display a muramidase-like activity *in vitro* and cleave polymeric Lys-type PGN chains[38]. One model with some experimental support is that GNBP1 cleaves PGN into shorter dimeric or tetrameric muropeptides that bind to PGRP-SA[38]. Indeed, GNBP1-digested Lys-type PGN can induce the Toll pathway in a GNBP1-independent, but PGRP-SA-dependent manner[38,39]. This presentation of Lys-type PGN to PGRP-SA by GNBP1 takes place within a tripartite GNBP1–PGRP-SA–PGN complex. However, overexpression of a PGRP-SA–GNBP1 complex in the absence of Lys-type PGN is sufficient to trigger Toll-pathway activation in the absence of infection, thus underscoring the importance of the signalling activity of this complex[34]. This model will be fully tested when mutants that affect the putative muramidase-like activity and not the signalling function of GNBP1 are characterized.

As illustrated by the PGRP-LC–PGRP-LE interaction, an important property of *D. melanogaster* pattern-recognition receptors (PRRs) is their ability to form various complexes to detect various microbial species. PGRP-SA cooperates with PGRP-SD for the detection of some Lys-type PGN bacteria, as the combination of a *PGRP-SD* mutation with either a *PGRP-SA* or a *GNBP1* mutation leads to an increased sensitivity to infections with *Streptococcus pyogenes*, whereas no strong phenotype is detected for either *PGRP-SA* or *PGRP-SD* mutants alone[33]. Also, activation of the Toll pathway by *Staphylococcus aureus* is almost normal in *PGRP-SA* or *PGRP-SD* mutants, but is strongly affected in the double mutants (TABLE 1). Therefore, the combination of several PRRs can expand the repertoire of microorganisms that are detected by the *D. melanogaster* immune system. The mechanistic basis for this observation is not understood at present.

Recognition of fungal infections. The Toll pathway is also preferentially triggered by fungi, as monitored by the sustained induction of *Drosomycin*[10,40]. Fungi are detected by a dual sensing system that converges with the proteolytic maturation of the Toll ligand Spätzle[9]. GNBP3 has a central role in the detection of components of the fungal cell wall, mainly by recognizing glucans. Therefore, similiar to PGRPs, GNBP family members can discriminate between distinct classes of microorganisms. GNBP3 seems to be a bona fide fungal PRR as it is required for host defence against yeast infections and for induction of the Toll pathway by killed yeasts; a GNBP3 recombinant protein binds to β-(1,3)-glucans, which are found in the fungal cell wall, and *GNBP3* is required for activation of the Toll pathway by alkali-treated preparations of fungal cell wall[9]. Furthermore, the overexpression of *GNBP3* in the absence of infection is sufficient to trigger the Toll pathway[9]. This basic sensing mechanism seems to be ancestral, as GNBP3 homologues are found in lepidopteran and hymenopteran insects, which shared a common ancestor with *D. melanogaster* 420 millions years ago[36].

A second sensing system was recently discovered that is independent of GNBP3 (REF. 9). Entomopathogenic fungi have developed strategies to penetrate the host by enzymatically and mechanically boring a hole through

Gram-negative binding proteins
(GNBPs; also known as βGRPs). This family is characterized by the existence of an N-terminal glucan-binding domain and a C-terminal domain with similarities to bacterial glucanases. GNBPs are present in most invertebrates, including deuterostomes, such as the sea urchin *Strongylocentrotus purpuratus*, but has not been found in vertebrates.

Pattern-recognition receptor
A germ-line encoded receptor that recognizes unique and essential structures that are present in microorganisms, but absent from the host. In vertebrates, signalling through these receptors leads to the production of pro-inflammatory cytokines and chemokines and to the expression of co-stimulatory molecules by antigen-presenting cells. The expression of co-stimulatory molecules, together with the presentation of antigenic peptides, by antigen-presenting cells couples innate immune recognition of pathogens with the activation of adaptive immune responses.

the insect cuticle. A fungal protease used by the entomopathogenic fungus *Beauveria bassiana* to digest the cuticle was shown to activate the Toll pathway by inducing the maturation of Persephone[41], a *D. melanogaster* haemolymph zymogen, into an active protease[9]. Persephone then triggers a proteolytic cascade that activates the Toll ligand Spätzle. Therefore, *D. melanogaster* does not rely solely on PRRs to detect infections, but can also detect the activity of virulence factors. Future studies will determine if this newly identified strategy for sensing microbial infections is as widespread in animals as it is in plants[42].

Activation of the Toll receptor by Gram-positive bacteria and fungi. Unlike some mammalian TLRs, the Toll receptor in *D. melanogaster* is not activated by interacting directly with microbial ligands[43]. Instead, the ectodomain of this transmembrane receptor binds to a cleaved form of the cytokine Spätzle, which is a protein that is structurally related to neurotrophins and is secreted in the haemolymph[44–46] (FIG. 3). As in mammals, the insect genomes encode a family of Toll receptors; in *D. melanogaster* this family comprises nine members[47]. Eight of these receptors (Toll, 18-wheeler and Toll3–Toll8) share sequence characteristics, both in the ectodomain and in the intracytoplasmic tail, that differentiate them from mammalian TLRs. Such differences might reflect a function of these *D. melanogaster* Toll receptors as cytokine receptors (the Spätzle family comprises six members[48]), rather than as PRRs. The sequence of Toll9 is divergent from that of the other eight *D. melanogaster* Toll receptors, and most closely resembles that of mammalian TLRs. An immune function for the *D. melanogaster* Toll receptors, with the remarkable exception of Toll itself, has not been demonstrated so far. Their expression patterns during embryogenesis and metamorphosis suggest that they have developmental roles[49]. Interestingly, the recent sequencing of the sea-urchin genome also revealed the existence of two categories of Toll receptors, with one subfamily (219 members) sharing similarities with vertebrate TLRs (and *D. melanogaster* Toll9), and another subfamily (3 members) sharing similarities with the eight conserved *D. melanogaster* Toll receptors[50]. It is possible that the common ancestor of vertebrates and invertebrates contained both forms of Toll receptor, and that the invertebrate form was lost in the vertebrate lineage, perhaps as a consequence of the acquisition of another type of Toll/IL-1R (TIR)-domain-containing cytokine receptor (such as IL-1R or IL-18R).

Spätzle is synthesized as an inactive dimeric precursor, linked by a disulphide bridge. The precursor is unable to bind and activate Toll and requires proteolytic processing of its 106 amino-acid C-terminus fragment (C106). This processed active form of the cytokine binds to Toll, and triggers signalling[44,45]. Two serine proteases have been identified that can cleave Spätzle and activate Toll signalling. The first, Easter, activates Spätzle in the perivitelline fluid on the ventral side of the *D. melanogaster* embryo[51], and provides an essential cue for the differentiation of the dorso–ventral axis of the embryo. The second, SPE (Spätzle processing enzyme), is activated at later stages of development in response to infection[52]. These two proteases activate Spätzle in response to different stimuli and are both secreted as inactive zymogens with an amino-terminal CLIP domain[115].

The link between SPE and the receptors that recognize fungal or bacterial products, or virulence factors, has not been defined in detail to date. For example, it remains to be determined whether Persephone, which is also a CLIP-domain serine protease, activates SPE directly. Several additional proteases have recently been suggested to participate in the activation of the Toll pathway[53]. One model proposes that distinct cascades of proteases are activated by fungi and Gram-positive bacteria, respectively[53]. An alternative possibility is that one cascade of proteases is activated by proteases secreted from virulent infectious microorganisms, as recently shown for *B. bassiana*[9], whereas another cascade is initiated by PRRs (such as PGRP-SA, PGRP-SD, GNBP1 and GNBP3) following activation by microbial ligands such as Lys-type PGN or β-glucans. Characterization of the phenotype of flies deficient for the genes encoding these CLIP-domain proteases will clarify this issue.

The Toll and IMD signalling cascades

The Toll pathway. Most of our information on Toll intracellular signalling derives from initial studies of the *D. melanogaster* embryo. One main difference between the Toll signalling pathway in embryos and in adults is the NF-κB-related transcription factor that is activated: whereas the output of Toll signalling is mediated by Dorsal in the embryo, the closely related factor DIF is the main regulator of antimicrobial peptide expression through Toll signalling in adult flies[40,54]. Here, we integrate data from studies of embryos and adult flies, although all aspects of signalling have not been verified at both stages of development.

Following cleavage by SPE, the dimeric cytokine Spätzle binds with high affinity to the N-terminal, extracytoplasmic region of Toll, and crosslinks the two Toll ectodomains[44] (FIG. 3). This binding triggers conformational changes in the receptor that lead to signalling. Signalling by Toll receptors, both in mammals and flies, involves the assembly of a multivalent complex around the intracytoplasmic tail of the receptor. This intracytoplasmic tail contains a 150-amino-acid TIR domain. The corner stone of this Toll-induced signalling complex (TISC) is the cytoplasmic adaptor MyD88 (myeloid differentiation primary-response gene 88), which is conserved in vertebrates and invertebrates, and is composed of a death domain (DD) and a TIR domain[55,56]. The TISC in *D. melanogaster* also contains two other DD-containing proteins — Tube, which has a bivalent DD, and Pelle, which is a member of the IL-1R-associated kinase (IRAK) family of serine-threonine kinases. *D. melanogaster* MyD88 and Tube form a membrane-bound pre-signalling complex through their DDs that is recruited to the Toll receptor following its activation by Spätzle. The formation of a MyD88–Tube–Pelle complex is mediated by the bivalent DD of Tube and leads to the activation of Pelle kinase activity[56].

Zymogen
The inactive precursor of a protease. The zymogen contains an amino-terminal pro-domain that keeps the protease in an inactive state. The removal of the pro-domain by another protease or by autoproteolysis leads to a conformational change that exposes the active site.

CLIP domain
CLIP domains are disulphide-knotted protein–protein interaction domains that are present in several invertebrate serine proteases and mediate sequential activation of immune zymogen cascades. The topology of the knot formed by the disulphide bonds is reminiscent of that of a paper clip, hence its name.

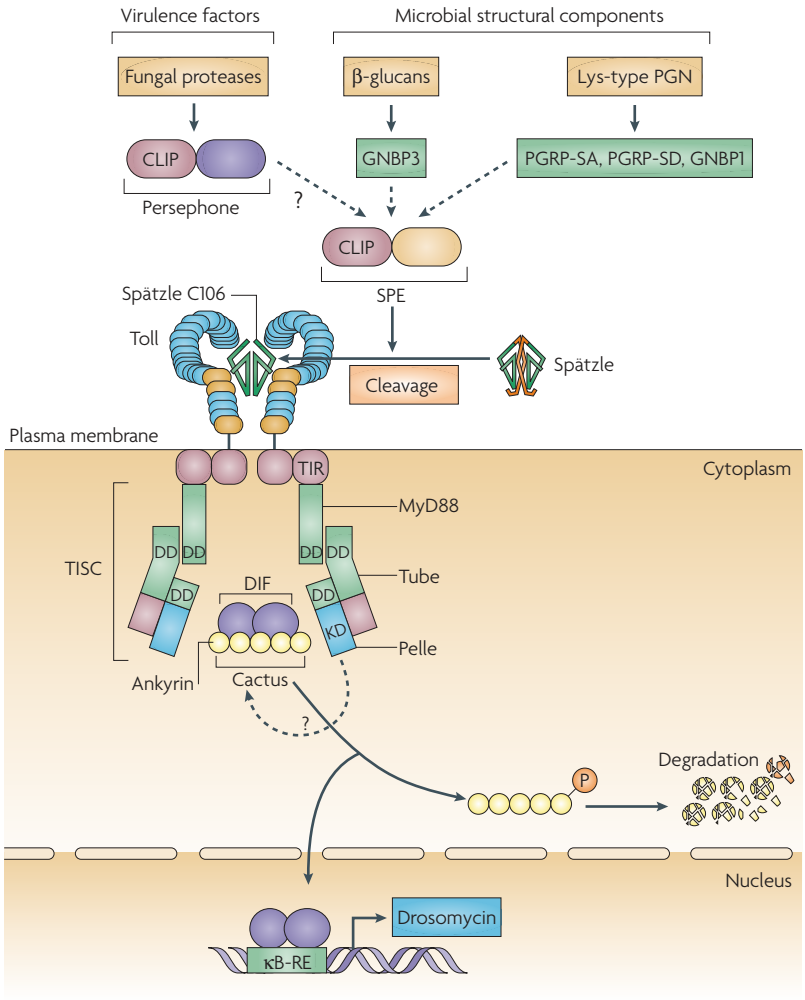


Figure 3 | **The Toll pathway in adult *Drosophila melanogaster*.** Infection is detected by soluble receptors in the haemolymph. Some of these receptors, namely peptidoglycan-recognition protein-SA (PGRP-SA), PGRP-SD and Gram-negative binding protein 1 (GNBP1), recognize Lys-type peptidoglycan (PGN) from Gram-positive bacteria, and other receptors (namely GNBP3) recognize β-glucans from yeast. Alternatively, the pathway can also be activated by fungal proteases, which are virulence factors and are detected as danger signals. Recognition of infection (either non-self or danger) triggers proteolytic cascades involving serial activation of CLIP-domain serine proteases, such as Persephone. The cascades activated by these pathogen-recognition receptors (PRRs) and virulence factors are distinct, as exemplified by the *Persephone* loss-of-function phenotype, (defective sensing of live entomopathogenic fungi, but not of killed fungi). The ultimate protease of the cascade, SPE (Spätzle processing enzyme), cleaves the precursor of the dimeric cytokine Spätzle. The released 106-amino-acid C-terminal fragment (known as Spätzle C106) binds to and activates the Toll receptor. Signalling through Toll involves the Toll-induced signalling complex (TISC), composed of three death-domain (DD)-containing proteins, MyD88 (myeloid differentiation primary-response gene 88), Tube and Pelle, which is the orthologue of mammalian IRAK (IL-1R-associated kinase). The trimeric complex assembles around the bipartite DD of Tube, which contains two opposite surfaces involved in the interaction with the DDs of MyD88 and Pelle. It is still unclear how the signal is transduced from the receptor complex to Cactus, a homologue of the mammalian inhibitor of NF-κB (IκB). One model that accounts for the existing data in the literature is that the Cactus–DIF (dorsal-related immunity factor; a homologue of mammalian nuclear factor-κB (NF-κB)) complex is recruited at the TISC, where Cactus may be phosphorylated by Pelle in a manner similar to the phosphorylation of interferon-regulatory factor 7 (IRF7) by IRAK1 in the Toll-like receptor 7 (TLR7) or TLR9 complex in mammals. Phosphorylated Cactus is rapidly polyubiquitylated and degraded, allowing for the nuclear translocation of DIF, and binding to NF-κB response elements (κB-RE), which in turn induces the expression of genes encoding antimicrobial peptides, such as *Drosomycin*. KD, kinase domain.

In contrast to vertebrates, *D. melanogaster* MyD88 does not seem to interact directly with the TIR domain of the Toll receptor in the embryo. Here, the zinc-finger adaptor Weckle is required to recruit the MyD88–Tube complex to the plasma membrane[57]. Weckle is not required for Toll signalling in adults and it is unclear whether the TISC in cells in the adult fat body contains other adaptor molecules.

Toll activation leads to the rapid phosphorylation and degradation of Cactus, a homologue of the mammalian inhibitor of NF-κB (IκB)[58,59], possibly through polyubiquitylation. This results in the graded release and nuclear translocation of the NF-κB transcription factors Dorsal (in embryos) or DIF (in adults). The mechanism by which activation of the TISC leads to phosphorylation of Cactus is still uncertain. By analogy with mammalian signalling, it was thought that Pelle might activate a TNFR-associated factor (TRAF)-related molecule, and/or kinases related to the mitogen-activated protein kinase kinase kinase (MAPKKK) and IκB kinase (IKK) families. However, such molecules have not been shown to have a role in Toll signalling in *D. melanogaster*[60,61]. One intriguing observation in the embryo is that Cactus-bound Dorsal can associate with the TISC through its interaction with Tube, potentially allowing the phosphorylation of Cactus by Pelle, therefore removing the need for an intermediator kinase[62–64]. Indeed, the activation of Dorsal and DIF could be mediated directly at the level of TISC, in a manner conceptually similar to the recently described activation of transcription factors of the IRF family by IRAK1 in mammals[65].

In addition to targeting the inhibitor Cactus to release Dorsal or DIF, signalling through the Toll receptor also results in the phosphorylation of Dorsal by an as yet unidentified kinase. This phosphorylation is required for nuclear import of the transcription factor, as shown by the fact that a mutant Dorsal protein that cannot interact with or be inhibited by Cactus still requires Toll signalling to efficiently reach the nucleus and regulate gene expression[66]. DIF might also require post-translational modifications for full activity. So, there seems to be a dual regulation of Dorsal (and possibly DIF) activity by Toll signalling, with a well-characterized basal level of activation regulated by the phosphorylation and degradation of Cactus, and a second level of activation, which is still poorly understood, acting directly on Dorsal or DIF that is essential for full stimulation of this pathway. During development, the Toll pathway is negatively regulated by the secreted factor WntD (Wnt inhibitor of Dorsal)[67,68]. However, WntD is not required in adult flies for the induction of *Drosomycin* expression in response to a Gram-positive bacterial infection[68].

The IMD pathway. The DD-containing adaptor IMD has a central role in the response to Gram-negative bacteria[69] (FIG. 4). Its DD has sequence similarity with that of mammalian receptor-interacting proteins (RIPs)[70]. The IMD adaptor mediates the action of the PGRP-LC intracytoplasmic domain, and possibly that of non-secreted

PGRP-LE when it functions as a putative intracellular receptor[15,20]. Both PGRPs contain a RIP homotypic interaction motif (RHIM)-like motif in their N-terminal domain that is required to initiate signalling[20]. It has been proposed that an unidentified adaptor mediates the interaction between PGRP-LC and IMD because a domain mediating an interaction between these two molecules is dispensable for signalling[20,71].

IMD functions as a signalling platform that initiates two genetically distinct processes that ultimately target the NF-κB-like transcription factor Relish, which is the main transcription factor of the IMD pathway[72]. The structure of Relish is similar to that of the mammalian p100 and p105 NF-κB precursors, with an N-terminal DNA-binding REL domain and C-terminal ankyrin repeats that are characteristic of the IκB family. After an immune challenge, a first process of the IMD pathway leads to the phosphorylation of Relish, which then triggers its cleavage by a second process[73,74]. The C-terminal ankyrin repeats remains in the cytoplasm, whereas the REL moiety migrates to the nucleus where it regulates the expression of genes encoding AMPs and many other proteins[30,74].

The first process, which involves phosphorylation of Relish by the IKK signalling complex formed by the catalytic subunit IRD5 (immune-response deficient 5; the fly homologue of mammalian IKKβ) and a regulatory subunit Kenny (the fly homologue of mammalian IKKγ), is thought to tag Relish for subsequent cleavage[60,61,73]. The genetic data available to date are consistent with this model of phosphorylation-dependent cleavage, but direct biochemical validation is still lacking. Activation of the IKK signalling complex is itself mediated by the MAPKKK transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1), which forms a complex with the regulatory subunit TAK1-binding protein 2 (TAB2)[75–79].

As in mammals, the activation of TAK1 and IKK probably involve K63-linked polyubiquitin conjugation, possibly as a scaffold for the assembly of active complexes[80,81]. In the absence of direct biochemical data, genetic evidence suggests that the ubiquitin conjugating E2 enzyme Bendless (which is similar to mammalian ubiquitin-conjugating enzyme 13 (UBC13)) and ubiquitin-conjugating enzyme E2 variant 1 (UEV1A) are required for the phosphorylation of Relish in S2 cells. Indeed, *Bendless* mutant flies display decreased induction of the IMD pathway target *Diptericin* after *E. coli* challenge[81]. Genetic analysis also indicates that the RING (really interesting new gene)-finger containing protein *D. melanogaster* inhibitor-of-apotosis protein 2 (DIAP2) is a candidate for the role of E3 ligase that mediates the last step of ubiquitin conjugation to as-yet-unidentified proteins of the IMD pathway[78,79,82,83], although it may also act at other steps in the pathway[82]. In summary, IKK complex activation in *D. melanogaster* and mammals exhibit striking similarities.

The second process, which is the cleavage of Relish, involves at least three genes; *imd*, *Fadd* (FAS-associated death domain), and *Dredd* (death-related ced-3/Nedd2-like protein). Upon immune challenge, IMD recruits the DD-containing adaptor FADD, which in turn interacts with the caspase-8 homologue DREDD[84]. This caspase is required for the cleavage of Relish[85,86], a process which does not involve the proteasome[74]. Immunoprecipitation experiments have shown the association of Relish with DREDD[87], but a direct demonstration of Relish cleavage by DREDD is still required. Unexpectedly, the FADD–DREDD complex is also required in the first process, upstream of TAK1, for activation of the IKK complex, and thus functions at two distinct steps in the pathway that leads to cleavage of Relish[81] (FIG. 4).

Besides initiating Relish signalling for at least a day, immune stimulation of the IMD pathway also activates the JUN N-terminal kinase (JNK) pathway, with immediate response kinetics over a few hours[88,89]. The branch-point occurs at the level of the TAK1–TAB2 complex[76,88], which potentially intersects a TNF-like signalling cascade controlled by Eiger and Wengen[77]. However, it is not clear whether Eiger-induced signalling takes place in the fat body as Eiger is mainly expressed in neural tissues, at least until late larval stages[90]. Whereas JNK signalling may briefly control the expression of some AMP genes, the physiological relevance of the short-lived activation of this MAPKKK pathway in the systemic host defence remains to be assessed[76,79,88,89,91,92].

In addition to the control of IMD pathway activation by digestion of immunogenic PGN by catalytic PGRPs, negative regulation of this pathway also takes place at the level of expression of Relish and DREDD and involves the ubiquitin–proteasome pathway and Caspar, a *D. melanogaster* homologue of the FAS-associating factor 1 (REFS 93,94). The absence of these negative regulators leads to either an activation of the IMD pathway in the absence of an immune challenge[93,94] or an increased response to an immune challenge[93].

We have so far discussed the Toll and IMD pathways separately. The NF-κB transcription factors activated by these pathways recognize distinct κB binding sites on the promoter of target genes[95]. Interestingly, some reports show that experimental challenge by various microbes can concomitantly activate both pathways. Septic injuries with filamentous fungi, which strongly activate the Toll pathway and expression of *Drosomycin*, also transiently induce *Diptericin* through PGRP-LC and the IMD pathway[9,10]. Induction of *Defensin* and genes encoding Cecropins and Attacins by a mixture of Gram-positive and Gram-negative bacteria was variably reduced in both *Toll* and *imd* loss-of-function mutants[3]. In keeping with this, concomitant stimulation of Toll and IMD pathway by agonists in S2 cells (through Spätzle C106 and DAP-type PGNs) results in stronger levels of induction of AMP gene expression as compared to single challenge with either agonist[96]. Taken together, these results suggest that the Toll and IMD pathways can act synergistically. It will be interesting to address in future studies whether concomitant activation of the Toll and IMD pathways by a given microorganism reflects the activation of distinct PRRs by the same microbial ligand (for example DAP-type binds to both PGRP-SA and PGRP-LC[37]), the presence of distinct microbial agonists

Polyubiquitylation
The attachment of the small protein ubiquitin to lysine residues present in other proteins. Protein ubiquitylation occurs in three enzymatic steps requiring a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). Subsequent ubiquitylation events can extend from the initial ubiquitin at one of its seven lysine residues (K6, K11, K27, K29, K33, K48 or K63) forming a polyubiquitin chain.

Receptor-interacting protein
(RIP). A family of serine/threonine kinases with homologous kinase domains. RIP1 is recruited to TNFR1 and mediates TNF-induced activation of JNK and NF-κB transcriptional pathways. RIP2 (CARDIAK/RICK) binds to caspase-1 and activates NF-κB.

RHIM motif
In mammals, the RHIM motif is found in the adaptor proteins TIR-domain-containing adaptor protein inducing IFNβ (TRIF), which is part of the TLR pathway, and RIP, which is part of both the TLR and TNFR pathways. This motif is required for the interaction between RIP and TRIF and subsequent NF-κB activation by TLR3.

K63-linked ubiquitylation
Formation of polyubiquitin chains on a target protein that are linked through the lysine at position 63 (K63) in ubiquitin. Unlike K48-linked chains, which are the principal signal for targeting substrates for proteasomal degradation, K63-linked ubiquitin-modified proteins regulate protein function, target certain proteins for endocytosis, and interact with proteins with specific ubiquitin-binding domains.

S2 cells
A cell-line established by Imogen Schneider (S2 stands for Schneider's line 2) in 1970, from *Drosophila* embryos. S2 cells are the most widely used *Drosophila* cell line. Of particular interest for the study of innate immunity, these cells share several properties with haemocytes, in particular the Toll- and IMD-mediated induction of AMPs, and have the capacity to phagocytose microorganisms.

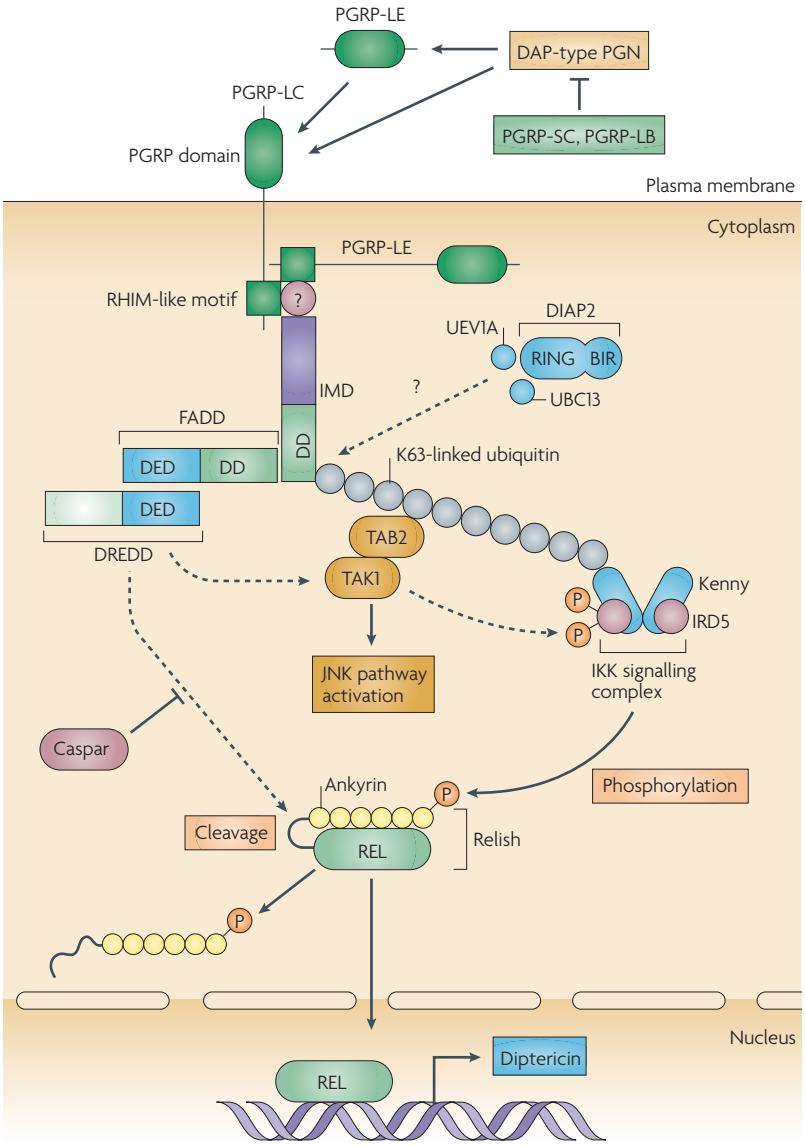


Figure 4 | **The IMD pathway in *Drosophila melanogaster*.** Peptidoglycan-recognition protein-LC (PGRP-LC), and potentially PGRP-LE, sense the presence of Gram-negative bacteria and, following multimerization, activate the immune deficiency (IMD) pathway through their RHIM (RIP (receptor-interacting protein) homotypic interaction motif)-like motifs. Because the PGRP domain that binds to IMD is dispensable for signalling, it has been predicted that the interaction between PGRP and IMD is mediated by an as yet unidentified protein. IMD has an essential role in controlling the phosphorylation of Relish through the activation of transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1) and IκB kinase (IKK) complexes and cleavage of Relish through the caspase homologue death-related ced-3/Nedd2-like protein (DREDD). TAK1 and IKK activation requires several proteins, including FAS-associated death domain (FADD), DREDD and molecules involved in the conjugation of Lys63 (K63)-linked polyubiquitin chains to unknown substrates. In this scheme, we speculate that IMD is one such substrate, as its orthologue in mammals, RIP1 (receptor-interacting protein 1), has been shown to be polyubiquitylated. The ligase function provided by the RING-finger of TRAF (tumour-necrosis-factor receptor (TNFR)-associated factor) in mammals may be mediated in *Drosophila melanogaster* by the RING-finger containing *D. melanogaster* inhibitor-of-apotosis protein 2 (DIAP2). TAB2 (TAK1-binding protein 2) contains a zinc finger that binds to K63-linked polyubiquitin chains and therefore might participate in the assembly of a Kenny–IRD5 (immune-response deficient 5) signalling complex. Again, an ability of the human homologue of Kenny, IKKγ, to bind to K63-linked polyubiquitin chains has been documented in mammals, but this has not yet been shown for Kenny in *D. melanogaster*. DD, death domain; DED, death effector domain; UEV1A, ubiquitin-containing enzyme E2 variant 1.

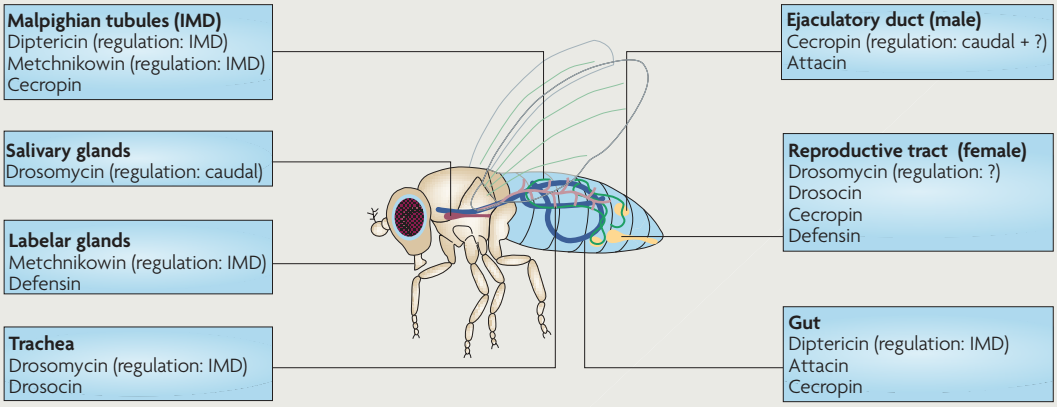
on the same microorganism, or the dual capacity of eliciting a response by microbial patterns and virulence factors such as proteases.

Expression and putative roles of effector proteins

Antimicrobial peptides. AMPs were the first immune effector molecules to be characterized in *D. melanogaster*. These molecules, or their orthologues from larger insect species, were initially identified using functional tests, and most have been shown to be active against many bacteria and/or fungi[1,2]. These molecules have since been used as convenient markers to monitor the activation of the Toll or IMD pathways, and there is a good correlation between the expression of some of these peptides and resistance to infection. For example, *Toll*-mutant flies fail to produce the antifungal peptides Drosomycin and Metchnikowin, and are susceptible to infections with the filamentous fungus *Aspergillus fumigatus*[3]. However, the exact contribution of AMPs to infection *in vivo* has not been addressed until recently. Constitutive expression of AMPs using a heterologous promoter can rescue to some extent the phenotype of flies mutant for the IMD and Toll pathways[97]. Ectopic expression of a *Defensin* transgene protects flies that are deficient for both Toll- and IMD-pathway activation against challenge by at least some Gram-positive bacteria, but not by Gram-negative bacteria or fungi. By contrast, ectopic expression of *Attacin* or *Drosomycin* transgenes leads to increased resistance of the double-mutant flies to infections with some Gram-negative bacteria or fungi, respectively. These results were recently extended to epithelial defences, as expression of *Cecropin* or *Diptericin* in the midgut of flies was shown to rescue mutations in the IMD pathway[98,99,124]. The main limitation of these experiments is the quantity of active peptides produced in transgenic flies, which might differ from the *in vivo* context of an infection. Mutations of the genes encoding AMPs are not available to test their exact roles in a reliable manner *in vivo*. However, studies in *Anopheles* mosquitoes have established that Defensin has a crucial role in the control of Gram-positive bacterial infections, but is dispensable for the control of infection by the Gram-negative bacterium *E. coli*, a result which is in good agreement with the data described for *D. melanogaster*[100].

Other effectors. In addition to the evidence that AMPs have a role in the control of bacterial and fungal infections, several lines of evidence indicate that the Toll and IMD pathways control other effector mechanisms, as illustrated by three examples below. First, the main antifungal peptide regulated by the Toll pathway, Drosomycin, is not active against *B. bassiana* (but is active against other filamentous fungi, such as *A. fumigatus*), in either *in vitro* assays, or *in vivo* experiments[97], indicating that other targets of the Toll pathway operate to counter infections with this fungus. Second, flies that are mutant for *kenny* (the homologue of IKKγ) are resistant to infection with Gram-positive bacteria, in the absence of induction of *Defensin* expression[61]. Third, *PGRP-SA* and *GNBP3* mutant flies are highly susceptible to infections with *S. aureus* and

Box 2 | **Immunity in barrier epithelia**



Drosophila melanogaster interacts continuously with microbes in its environment through the interfaces provided by contact epithelia in the digestive, respiratory and reproductive systems. LacZ and green fluorescent protein (GFP) reporter genes have revealed that several antimicrobial peptides (AMPs) are expressed in various tissues of laboratory-reared flies[120–122] as illustrated in the figure. Whereas some AMPs are constitutively expressed in the reproductive tract or salivary glands, others are induced through the immune deficiency (IMD) pathway by microbes in direct contact with the epithelium.

Multiple physical and immunological barriers of the digestive tract

The digestive tract is the primary source of contact between *D. melanogaster* and microbes. The midgut absorbs nutrients while preventing the entry of microorganisms by several mechanisms. The first layer of protection is a physical barrier, the chitinous peritrophic matrix that lines the midgut and is secreted by the proventriculus (cardia). Most microorganisms are blocked by this matrix, except for *Serratia marcescens*, which can traverse the intestinal epithelium[124]. The second level of protection relies on the local production of AMPs in the proventriculus and in the midgut. A third level of protection is provided by the production of reactive oxygen species (ROS) in response to the ingestion of large quantities of dead or live bacteria or yeasts[123]. The fly is protected from this oxidative shock by a catalase: in its absence, the flies succumb to an oral challenge with dead bacteria[107]. The enzyme responsible for the production of intestinal ROS is Duox, an NADPH oxidase that also contains a myeloperoxidase domain[123]. The mechanism of activation of this oxidative response is currently unknown and does not require either the Toll or IMD pathways. This response is less effective, however, against microorganisms that produce ROS protective enzymes such as catalases[99]. These resistant microorganisms are controlled by a local activation of the IMD pathway in the cardium and midgut[98,99,124]

B. bassiana, respectively, compared with wild-type flies, even though the Toll-pathway marker *Drosomycin* is induced to near wild-type levels in these flies[9,33].

Differential expression studies have shown that the expression of several hundreds of molecules is induced or upregulated by septic injury in addition to that of AMPs[30,101–104]. These genes encode proteins that are involved in microbial recognition, phagocytosis, melanization, reactive oxygen metabolism or iron sequestration. These include genes encoding the serpin Spn27A, which regulates the melanization cascade[105,106], and PGRP-LB, which has an essential role in downregulation of the IMD pathway[28]. Finally, the identification of a catalase induced by septic injury that maintains redox homeostasis in flies, has highlighted the role of reactive oxygen species in the control of gut infections in flies[107] (BOX 2).

Of note, a large fraction of the genes that are upregulated in response to sepsis have no known function to date. To understand how these induced molecules contribute to the control of infection will be challenging, as these molecules might act synergistically, or be specifically active on a selected subset of pathogens that infect flies. One possible approach to address this issue will be to compare the response to infectious bacteria, which somehow evade the immune system, with the response to innocuous non-pathogenic bacteria. Recent studies point to significant differences in the immune responses generated by bacteria that succeed or fail to establish a robust infection, and these differences might reveal novel strategies used by the host to counter infections[108,109].

Conclusions

The recent advances in the understanding of the molecular mechanisms involved in the recognition of typical Gram-positive and Gram-negative PGNs are spectacular and are not yet fully paralleled in the field of recognition of microbial inducers by the mammalian TLRs. Nevertheless, PGN structures are complex and diverse in the microbial world and it remains to be established how the repertoire of PRRs in the fly deals with this complexity. One lead is provided by the existence of several splice isoforms of the PGRP-LC receptors, as well as the combinatorial interactions between PRRs, to increase the range and sensitivity of detection of infections.

Surprisingly, in the insect immune system, only PGN and β-glucans have been firmly established as inducers of innate immunity to date. This contrasts with the variety

Redox homeostasis
The protection that is conferred to the fly against the toxic oxidative response that is triggered in the gut in response to the ingestion of high doses of microbes. This protection is mediated by immune regulated catalase (IRC), the transcription of which is also triggered in response to septic injury.

of microbial ligands, in addition to PGN and β-glucans, that activate innate immune responses in mammals (for example, LPS, lipopeptides, nucleic acids and proteins). Importantly, the two established recognition systems — PGRPs and GNBPs — activate signalling pathways that lead to gene reprogramming in response to infection. This is apparently not the case for other recognition proteins that can bind microbial cell-wall components, such as various lectins or scavenger receptors. In another context, some receptors, such as those belonging to the Eater family[110,111], can bind bacteria and promote their phagocytosis, a distinct outcome from that envisioned here.

An unexpected recent finding is that in addition to, or in parallel with, monitoring microorganisms through circulating or membrane-bound PRRs, adult flies can also detect the presence of a pathogen by sensing a virulence factor[9]. In a well documented case, this virulence factor is a protease secreted by an invading filamentous fungus, *B. bassiana*, but we speculate that this sensing system is of general relevance, as many microorganisms secrete proteases. Indeed, the study of host defence in plants has led to the identification of multiple, constantly evolving, devices that sense the activity of pathogen virulence factors[42].

Our understanding of the two signalling pathways that are activated during microbial infections has increased considerably in recent years, mainly through genetic analysis. There are clearly some similarities between the intracellular portion of the Toll signalling cascade and the IL-1R and TLR–MyD88 dependent pathways, although the *D. melanogaster* IKK complex does not seem to have a role in this pathway (FIG. 3) (see Supplementary information S3 (Figure)). The IMD pathway, by contrast, is evocative of both the TNFR and the TLR–MyD88-independent pathways (FIG. 4) (see Supplementary information S4 (Figure)). It is thought to involve complex ubiquitylation processes, which are still poorly understood. Of great interest is the link between the IMD pathway, which was initially discovered for its role in AMP induction, and the JNK pathway. Clearly, the IMD pathway is considerably more complex than was anticipated a few years ago, and the genetic data now call for an in-depth analysis at the biochemical and cell-biology levels. From a fundamental standpoint, it is puzzling that the Toll pathway is required for host defence against highly dissimilar microorganisms: Gram-positive bacteria and eukaryotic fungal parasites. Similarly puzzling is the observation that activation of the Toll pathway is built on pathogen recognition by circulating PRRs and subsequent activation of a zymogen cascade, whereas activation of the IMD pathway is directly mediated by recognition of microbial patterns at the cell membrane level. High sensitivity of detection may be provided in the first case by the amplification of the signal provided by the zymogen cascade. As regards the IMD pathway, homo- or heterodimerization of PGRP-LC and PGRP-LE isoforms possibly increases sensitivity to microbial ligands.

Over the last few years, the role of phagocytosis — the initial host-defence mechanism discovered in invertebrates — has again come to the fore, as it may help contain pathogens that resist or avoid the systemic immune response. The discovery that the neuronal immunoglobulin superfamily member DSCAM, which is encoded by a gene that can potentially generate 18,000 splice isoforms, is expressed by haemocytes and by cells in the fat body has also raised considerable interest as to the possibility of generation of a large receptor repertoire in *D. melanogaster*[112]. Furthermore, induction of resistance to lethal doses of a pathogen by priming with sublethal doses was observed for *Streptococcus pneumoniae* and *B. bassiana* and required both the Toll pathway and phagocytosis[113]. In depth analysis of the mechanisms underlying these observations is among the challenges of the field. Pending such studies, great care is warranted in the interpretation of these results and in drawing provocative parallels with mammalian lymphocyte-dependent adaptive immune defences.

We hope that the studies of *D. melanogaster* immunity, which have on some occasions, such as those regarding Toll receptors, been so fertile for the study of innate immunity in general, will continue to be at the vanguard of our study of the fight between microorganisms and their hosts. Indeed, whole genome screens in cell lines or flies, and infection models using insect or human pathogens will provide further advances (BOX 1). The key advantage of *D. melanogaster* in this context is that, in the absence of an adaptive response, innate immunity can be studied as an integrated system, at the level of the whole organism. These studies are now being extended to flies caught in the wild[114] and concomitantly to the identification and study of the natural and commensal pathogens that have shaped the *D. melanogaster* host defences.

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DATABASES
FlyBase: http://flybase.bio.indiana.edu/
Cactus | DIF | Defensin | Dorsal | Drosomycin | GNBP1 | GNBP3 | imd | Pelle | PGRP-LC | Relish | Spätzle | Toll | Tube

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