How *Drosophila* combats microbial infection: a model to study innate immunity and host–pathogen interactions

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During the past year, dramatic progress has been achieved in our understanding of *Drosophila* immune reactions. The completion of the *Drosophila* genome sequencing project, microarray analysis and the use of genetic screens have led to the identification of several new genes required to combat microbial infection, filling in some important gaps in the understanding of innate immunity. At the same time, this insect was used as a model for the study of host–pathogen interactions. The recent major advances on the mechanisms by which this insect defends itself against intrusion of pathogens are discussed in this review.

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Abbreviations

AMP	antimicrobial peptide
GNBP	Gram-negative-binding protein
JAK	janus kinase
Imd	immune deficiency
LPS	lipopolysaccharide
PAMP	pathogen-associated molecular pattern
PGRP	peptidoglycan recognition protein
STAT	signal transducer and activator of transcription
TEP	thiol ester protein
TLR	Toll-like receptor
TNF	tumor necrosis factor

Introduction

The fruit fly, *Drosophila*, spends its entire life cycle in decaying organic matter, such as injured or rotting fruit. In such an environment enriched in microorganisms, *Drosophila* often acts as a vector for microbial infection — adult flies transmit yeast and bacteria from one plant to another while the larvae deliver them deeper into the fruit (Figure 1). It is, therefore, not surprising that, during the four stages of the *Drosphila* life cycle, this insect uses efficient mechanisms to prevent microbial infection (reviewed in [1–3]).

First of all, the external cuticle offers an effective physical barrier against the penetration of microbes. In addition, both the gut and trachea, which are two main routes of infection, are lined with chitinous membranes. An environment hostile to microbial colonisation is maintained in the gut by its low pH and by secretion of antimicrobial factors such as lysosymes. When pathogens breach these physical and chemical barriers, they activate a wide range of inducible immune reactions (Figure 2). Firstly, breakage of the cuticle after injury or microbial infection induces rapid proteolytic cascades that lead to blood clotting and melanization (see later for a definition). Secondly, a cellular immune response that involves different types of haemocytes (blood cells), which participate in pathogen clearance by phagocytosis or by encapsulating larger parasites, is mounted. Finally, during systemic infection, a large set of inducible effector molecules, such as antimicrobial peptides (AMPs), stress response proteins and other factors required for opsonization and iron sequestration, are produced mainly by the fat body and secreted into the blood. As illustrated in Figure 2, the *Drosophila* immune responses are interconnected and synergistic in their effects. In this review, we discuss the recent major advances on the mechanisms by which *Drosophila* defends itself against intrusion by pathogens.

Haemocyte differentiation and function

Insects possess an open circulatory system that contains the haemolymph (the insect blood), which is pumped by a basic heart called the dorsal vessel. The haemolymph does not play a role in oxygen transport but is the major site of resistance during systemic infection. *Drosophila* larvae and adults contain a few thousand blood cells, which can be divided into the following four types on the basis of structural and functional features: secretory cells; plasmatocytes (the most numerous cells, essentially phagocytic); lamellocytes (required for encapsulation of parasites); and crystal cells (involved in the melanization process) [4,5,6••]. *Drosophila* haematopoiesis occurs in two major phases. The first population of blood cells appears during embryogenesis in the anterior mesoderm. These cells then rapidly colonise the whole embryo [7]. One of the functions of embryonic blood is to ingest apoptotic cells by phagocytosis. In addition, it has also been shown that blood cells have the capacity to engulf microbes injected into the embryo [8]. Toward the end of embryogenesis, the lymph glands (the larval haematopoietic organ) differentiate along the anterior portion of the dorsal vessel (Figure 3a). This organ, which disappears during metamorphosis, contains most haemocyte precursors, therefore serving as a haemocyte reservoir [6••]. In larvae, haemocytes can circulate freely in the haemolymph, but a large fraction of them, the sessile haemocytes, are attached to tissue [6••]. Four genes, *serpent*, *lozenge*, *U-shape* and *glial cells missing,* that regulate key steps of *Drosophila* hematopoietic lineage commitment have recently been identified (Figure 3b) [9,10••,11]. Given the similarities between Serpent, Lozenge and U-shape to mammalian haematopoietic factor GATA, Acute Myeloid Leukemia-1 (AML1) and Friend of GATA (FOG), respectively, these studies strongly suggest conservation of the molecular basis for blood cell lineage in mammalian and *Drosophila* haematopoeisis [9,10••,11]. The differentiation

Figure 1

Drosophila and microorganisms co-exist in numerous ways. *Drosophila* larvae and adults develop in decaying organic matter (the figure shows an injured *Opuntia* fruit) and often serve as vectors for microbes. Photograph appears courtesy of J Rouault.

and proliferation of larval haemocytes occur either in the lymph gland or in circulation. These processes are under the control of two conserved signalling pathways: the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, which is important for lamellocyte differentiation [12]; and the Toll pathway, which is required for proper haemocyte proliferation [13].

So far, we have little information on the mechanisms that underlie phagocytosis and encapsulation in *Drosophila*. A functional study using a dsRNA knockout in cultured cells of the mosquito *Anopheles gambiae*, however, indicates the role of a humoral protein called *Anopheles* thiol ester protein 1 (aTEP-1) in opsonization [14••]. This study suggests that aTEP-1 binds to the surface of both Gram-negative and Gram-positive bacteria and promotes phagocytosis by haemocytes. Interestingly, the *Drosophila* genome encodes four TEP-encoding genes that may play similar functions [15•]. Given the sequence similarities between TEP and vertebrate complement factors C3 and α-2-macroglobulin, these studies point to the ancient origin of complement-like protein in promoting phagocytosis [14••].

Coagulation and melanization

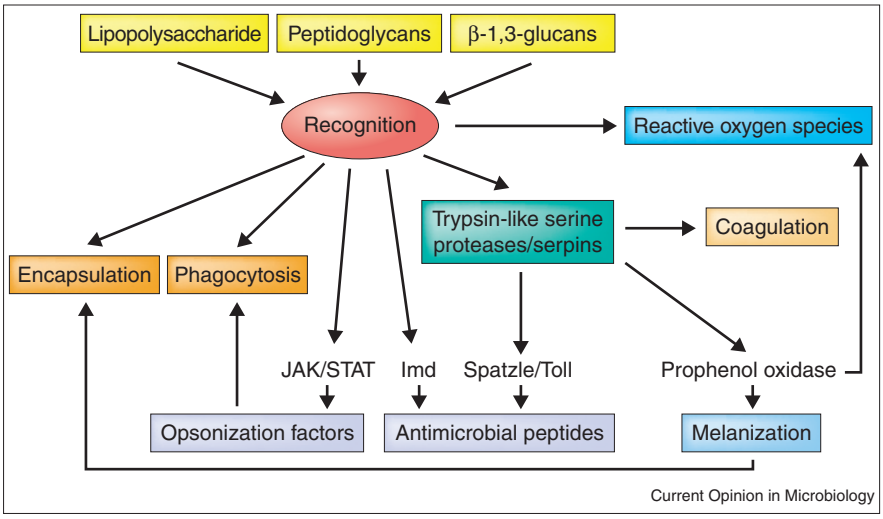
As we mentioned above, breakage of the host cuticle in invertebrates immediately induces the clotting of blood and melanization at the injury site [16,17]. These two reactions are essential to limit the spread of microbes during systemic infection. Melanization is a common defence mechanism among invertebrates and, in addition to wound healing, it is associated with encapsulation [17]. It requires the activation of phenoloxydase, an enzyme that catalyses the conversion of dopamine to melanin, which is toxic for microorganisms. This reaction also leads to the production of cytotoxic reactive oxygen species (ROS) that may play a role in combating infection.

The coagulation and melanization reactions are poorly studied in *Drosophila*. However, studies performed in other arthropods indicate that these cascades are triggered after recognition of microbial elicitors, such as lipopolysaccharide (LPS) or β-1,3-glucan, via a serine protease proteolytic cascade [17,18]. Interestingly, the *Drosophila* genome contains a large number of genes that encode serine proteases, protease inhibitors and other metabolic enzymes that may be involved in coagulation and melanization [2]. The observation that some of these genes are upregulated after microbial infection supports this assumption of their roles during the immune response [19••].

The fat body and the humoral response

One of the landmarks of the *Drosophila* immune response is the synthesis by the fat body of several antimicrobial peptides with distinct but overlapping specificity ([1,20]; Figure 3b). The fat body tissue originates from the mesoderm during embryogenesis and becomes immunocompetent only at the onset of the larval stage [21]. Unexpectedly, a recent report shows that, although embryos are well-protected by a thick chorion, they can still express the AMP gene *Cecropin A* upon injection of LPS or Gram-negative bacteria [22•]. The expression of this gene, however, does not occur in the fat body but, rather, in the yolk nuclei at the early embryonic stage and in the epidermis at later stages. During the larval stage, the

Figure 2



Schematic overview of the *Drosophila* host defence. Detection of microbial pathogens (microbial elicitors indicated by yellow boxes) by recognition proteins (indicated by the orange ellipse) activates a large array of interconnected and synergistic host-defence mechanisms (indicated by other colored boxes).

size of the fat body cells increases dramatically by polyploidization and their immune competence increases under the control of ecdysone [23,24]. In the early adult stage, the larval fat body desegregates and is replaced by a new adult fat body with slightly different immune properties.

In some ways, the insect fat body plays a role similar to mammalian liver during the acute-phase immune response. The large size and the position of this tissue in the insect body cavity make the fat body a powerful machine that enables the secretion of peptides into the haemolymph and ensures that they rapidly reach their effective concentrations. The relevance of AMPs in the immune response of *Drosophila* is supported by the high susceptibility to infection of mutants that do not express AMP-encoding genes [25,26]. AMP production is only a subset of the humoral response; other induced humoral factors are likely produced in this tissue as well. So far, we still have little idea of how the fat body cells are informed to induce AMPs and how these peptides are processed and secreted.

Signalling cascades that regulate the humoral response after systemic infection

Toll and Imd pathways

Several recent studies have greatly enhanced our understanding of the *Drosophila* humoral response by characterizing new components that regulate expression of AMP-encoding genes in the fat body (for a review, see [27,28]). The current model is that each AMP-encoding gene is regulated by a balanced activity of two distinct signal transduction pathways: the Toll pathway, which is largely activated by fungal infection and Gram-positive bacteria, and the immune deficiency (Imd) pathway, which is mainly activated by Gram-negative bacteria (Figure 4). Many components of the Toll pathway were previously identified to be mutations that affect embryonic dorsoventral patterning [29]. By contrast, factors that function in the Imd pathway to regulate antibacterial responses remained largely unknown until the past year. Recently, several studies have led to the genetic and molecular identification of six components of the Imd pathway: Imd, dTAK1, Ird5, Kenny, the Dredd caspase and the rel factor Relish [26,30,31,32••,33,34•–37•,38••,39••,40]. The Imd and Toll pathways do not appear to share any intermediate components and mediate differential expression of AMP-encoding genes via distinct NF-κB-like transcription factors [41]. These pathways exhibit striking similarities with the Toll-like receptor (TLR) and tumor necrosis factor (TNF) cascades that regulate NF-κB activity in vertebrates, suggesting common evolutionary roots for the immune response pathways (discussed in [27,28]). However, flies use two distinct pathways for controlling distinct NF-κB proteins, whereas, in mammals, both the TLR and TNF pathways converge to the activation of the IKK complex, which regulates NF-κB factors. The use of two pathways in fat body cells to regulate these AMP-encoding genes may be an efficient mechanism to adapt the antimicrobial response to different aggressors by producing a specific subset of AMPs [34•,38••,41,42]. The mechanisms by which the promoter of each AMP-encoding gene integrates signals from the two pathways are not clear; other molecules, such as the GATA factor Serpent, may play important roles [43]. Moreover, several studies indicate some differences in the regulation of *Drosophila* AMP-encoding gene expression, depending on the developmental stage or the tissue, revealing the complexity of the regulation of AMP-encoding genes [43,44,45•,46].

In addition to several developmental functions, the Toll pathway regulates the antifungal response and is required for proper haemocyte proliferation [13], as described above. Therefore, Toll activation leads to a coordinated immune response that comprises both cellular and humoral

Figure 3 legend

Immune-responsive tissues in larvae. **(a)** The lymph gland is located along the dorsal vessel of the larva and can be considered to be a reservoir of haemocytes. **(b)** Four types of haemocyte – secretory cells, plasmatocytes, crystal cells and lamellocytes – have been identified to differentiate from a pool of stem cells. Haemocyte differentiation can take place both in the lymph gland and in circulation and is under the control of conserved regulators or signalling cascades. Parts (a,b) reproduced, with permission, from [4,6••]. *srp*, *serpent*; *lz*, *lozenge*; *gcm, glial cell missing*. **(c)** The site of expression of antimicrobial peptides (AMPs) in the larvae is indicated. Systemic infection induces a strong expression of seven types of AMP-encoding genes (plus isoforms) in the fat body. Local infection triggers the expression of a subset of AMP-encoding genes in several epithelia [45•]. Part (c) appears courtesy of JL Imler.

response. In contrast, the Imd pathway is dispensable for proper development and cellular immune responses. Several studies suggest that, besides its role in antibacterial peptide gene regulations, the Imd cascade may be involved in apoptosis [39••,47,48].

JAK/STAT

Similar to the Toll cascade, the *Drosophila* JAK/STAT pathway is involved in multiple developmental events and regulates the cellular immune response [12]. This cascade is not essential for the activation of AMP-encoding genes in *Drosophila*, although studies in mosquitoes have shown that *A. gambiae* STAT (AgSTAT) is translocated in the nucleus of fat body cells of bacterially infected mosquitoes, suggesting that this cascade controls other genes encoding humoral factors [49]. More recently, the gene encoding the complement-like protein TEP1 has been identified as a target of the JAK/STAT pathway in *Drosophila* [15•]. The stimulus that activates the JAK/STAT pathway is not yet known, but there is evidence that this pathway can be activated by the Toll cascade [15•].

Local versus systemic immune responses

The use of green fluorescent protein (GFP) reporter transgenes has revealed that AMP-encoding genes, in addition to the fat body, can also be expressed in several surface epithelia that are potentially in contact with microorganisms ([44,45•]; Figure 3c). These include the epidermis, the reproductive system, the respiratory tract and the digestive tract that are in contact with the external environment [21,44,45•]. Preliminary studies indicate a predominant role of the Imd pathway in the control of expression of AMP-encoding genes in these tissues [21,45•], but the mechanisms that regulate the tissue specificity of this local immune response remain to be explored. Epithelial expression of AMPs appears to be a general feature of multicellular organisms, given that, in both vertebrates and plants, AMPs also play a critical role in the local response to infection.

Recognition of pathogens

In *Drosophila*, the mechanisms for detecting infectious microbes are largely unknown. The current view is that some receptors can recognise surface determinants (pathogen-associated molecular patterns, PAMPs) that are conserved among microbes but absent in the host. These PAMPs include LPS, peptidoglycan and mannans [50]. After recognition, the PAMP receptors may stimulate immune responses by activating extracellular proteolytic

Figure 3

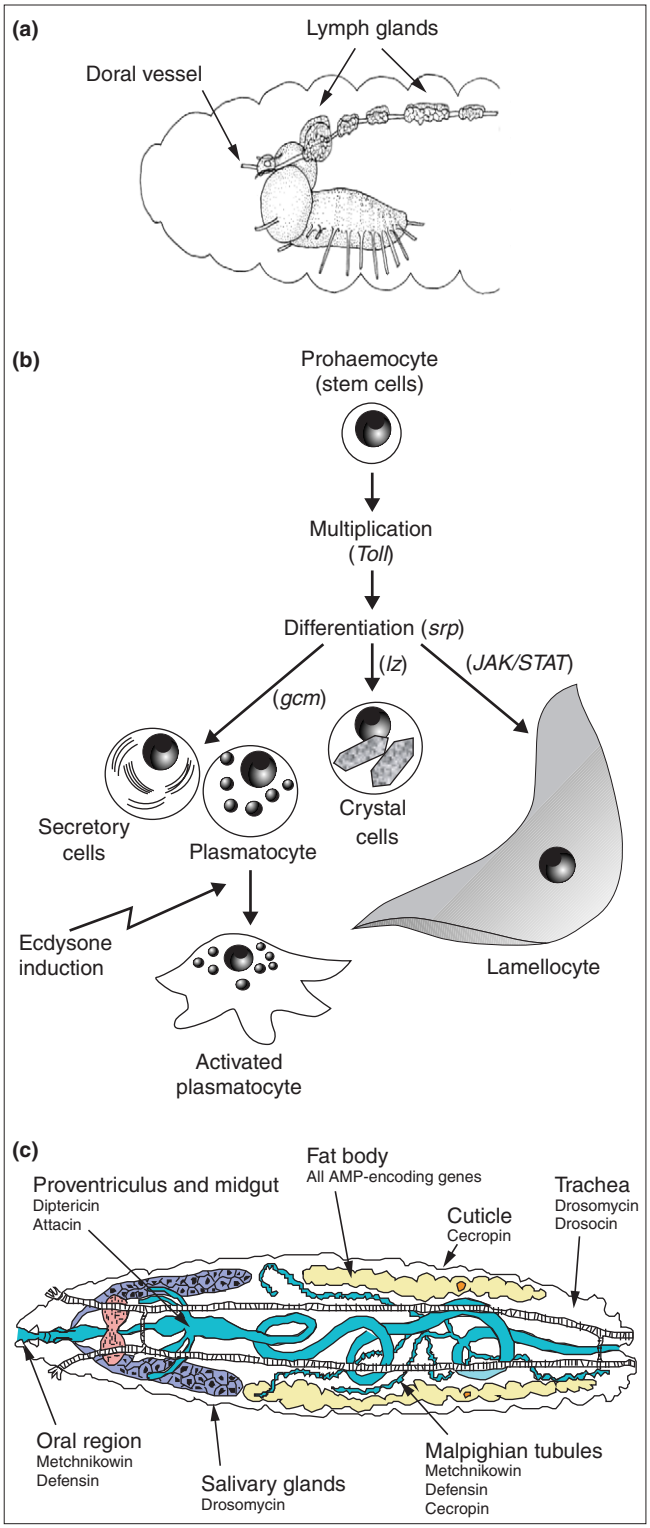
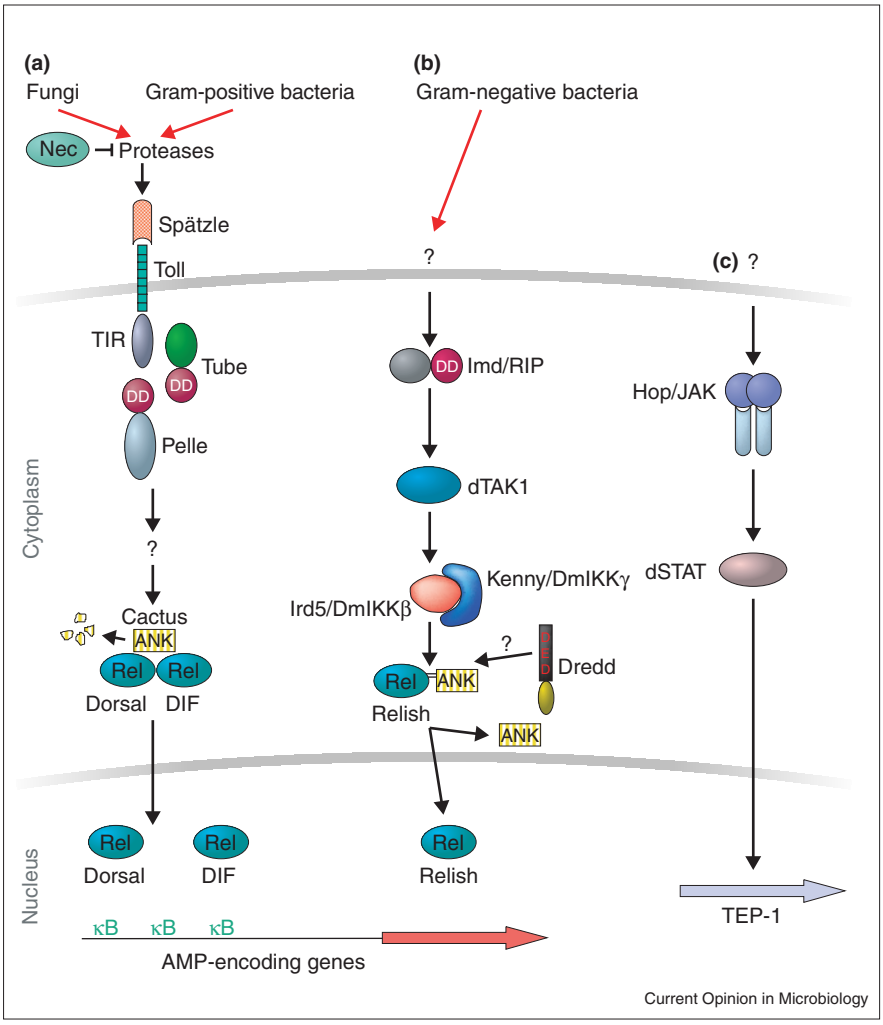


Figure 4



The Toll, Imd and JAK/STAT pathways control the synthesis of acute-phase proteins in the fat body. **(a,b)** AMP-encoding genes are regulated by two signal transduction pathways: the Toll pathway and the Imd pathway. (a) In the Toll pathway, an unknown proteolytic cascade that involves the serpin Necrotic is activated upon infection and leads to processing of Spätzle [51]. Binding of Spätzle on Toll activates an intracellular signalling cascade that leads to degradation of the IκB-like protein, Cactus, and nuclear translocation of the rel proteins Dif and Dorsal. (b) Relish is a compound rel/NF-κB transactivator related to P105 [26]. In the Imd pathway, this protein is cleaved upon microbial infection and cleavage is dependent on the caspase Dredd and the IKK complex (Ird5 and Kenny) [32••,33,34•–37•,40]. Epistatic studies suggest that the MAP3K dTAK1 function upstream of the IKK complex and downstream of Imd [38••,39••]. *imd* encodes a protein with a death domain similar to that of mammalian receptor-interacting protein (RIP) [39••]. For more details, see [27,28]. Note that the recognition mechanisms (indicated by a question mark) that target this cascade remain uncharacterized. ANK, ankyrin domain. **(c)** In the JAK/STAT pathway, observations that the expression of TEP-1 is reduced in *JAK*/*Hopscotch* mutants [15•] and that STAT is translocated into the nucleus of fat-body cells of bacterially infected mosquitoes [49] suggest that this cascade also controls the humoral response of *Drosophila*. Note that the recognition mechanisms (indicated by a question mark) that target this cascade remain uncharacterized.

cascades in the haemolymph and intracellular signalling pathways in immune-responsive tissues (Figure 2).

The *Drosophila* genome encodes a large number of proteins with putative recognition properties [2]. However, to date, there is no genetic demonstration of a role for these proteins in the activation of the immune response. Even though it is now clear that the Toll and Imd pathways are selectively activated in response to different microbes, the recognition proteins that target these pathways are still unknown [27]. In contrast to some of the mammalian TLRs, *Drosophila* Toll does not appear to function as a direct sensor of microbial compounds, but is activated via an unknown proteolytic cascade by a small cytokine-like molecule termed Spätzle [25,51]. The receptors that activate the Imd pathway in response to Gram-negative bacteria have not been identified. Given that TLR4 mediates recognition of Gram-negative bacteria in mice [52], it is tempting to speculate that one of the eight other Tolls encoded by the *Drosophila* genome may function in microbial recognition in the Imd pathway. However, expression of seven of the eight Toll homologues in cultured cells did not provide a clear demonstration of their function in the antibacterial defence [53].

Recently, two families of proteins have been implicated in pathogen recognition in *Drosophila*. Peptidoglycan recognition protein (PGRP) was identified as a Gram-positive-binding protein present in the moth *Trichoplusia ni* [54]. PGRP has also been implicated in the activation of the prophenoloxydase cascade in the silkworm *Bombyx mori* [55]. Interestingly, the *Drosophila* genome encodes 12 *PGRP* genes, and several of them are upregulated after septic injury [56]. In contrast to PGRP, Gram-negative-binding proteins (GNBPs) are only found in invertebrates and have been isolated as proteins that bind to LPS and β-1,3-glucan [57]. The *Drosophila* genome encodes three GNBPs and two immune-inducible-related proteins [19••,58]. Overexpression of *dGNBP-1* enhances LPS induction of AMP-encoding genes in cultured cells [58]. Furthermore, the observation that TEP molecules can bind to the surface of bacteria also suggests a role for these

proteins in pathogen recognition [14••]. In addition to PAMP detection, the insect may selectively recognize more specific determinants of pathogens, as shown in plant defence. Possibly, some of the signals that trigger the *Drosophila* immune response may involve other indirect mechanisms that sense the presence of pathogens through the breakage of the integument.

Monitoring the *Drosophila* immune response after different modes of infection

So far, most of our knowledge on the *Drosophila* immune response has been built upon the analysis of the host's reaction after injection of non-pathogenic bacteria in *Drosophila* larvae or adults. Recently, the use of oligonucleotide microarrays encompassing the full genome has revealed that several hundred genes out of 13 600 *Drosophila* genes are modulated after septic injury, confirming that this stimulus induces a wide-ranging response [19••]. The possibility of infecting a large number of animals in a short time and the high reproducibility of the *Drosophila* immune response after microbial injection have allowed the successful identification of key regulators of expression of AMP-encoding genes via systematic genetic mutagenesis [33,38••,59–61]. The application of similar screens will probably help to identify new factors involved in little-studied processes such as melanization and phagocytosis. In addition to the introduction of microbes into the body cavity, microbial injection results in an injury that leads to wound-healing reactions and to a stress response [62,63••]. The limitation of the injection approach is that it bypasses the first step of infection, which includes the attachment and entry of the pathogen. Recently, alternative approaches have been developed that use natural *Drosophila* pathogens and a natural mode of infection. The use of a natural mode of infection has contributed to the discovery of the ability of *Drosophila* to activate an immune response adapted to the invading organism [42], and enables the study of all the phases of infection from the initial interaction between the pathogen and its host to the activation of the immune response.

Beauveria bassiana is an entomopathogenic fungus that infects many insect species by penetrating their cuticle. Genetic experiments indicate that natural infection of *Drosophila* by this fungus leads to the expression, via the selective activation of the Toll pathway, of genes encoding antifungal activity [42]. Microarray analysis also confirms that infection by this fungus leads to a more specific immune response, compared with infection by bacterial injection [19••]. Recently, some strains of the phytopathogenic Gram-negative bacterium, *Erwinia carotovora*, which causes soft rot in fruit and uses insects as its vector, have been isolated for their capacity to trigger a systemic and local immune response in *Drosophila* larvae after gut infection [64•]. In this case, the immune response is largely mediated by the Imd pathway [38••,45•]. Finally, parasitoid wasps that inject their eggs into young instar larvae are among the most formidable pests to *Drosophila* in nature. Resistance to parasitoid infection is correlated with the capacity of the host to encapsulate the wasp eggs. The interactions between *Drosophila* and several wasp species provide a model to analyse cellular reactions [65–68].

Drosophila as a model host for studying human pathogens

The idea of using simple, genetically tractable host organisms to study the virulence mechanisms of human pathogens has recently emerged. Studies from several groups have clearly established the nematode *Caenorhabditis elegans* as an attractive model host for the study of *Pseudomonas aeruginosa* and *Salmonella typhimurium* pathogenesis [69,70]. *P. aeruginosa* has previously been shown to be highly pathogenic to *Drosophila* [71], and it is now the focus of several groups that use *Drosophila* as an alternative model system to study this important pathogen. *Drosophila* can be used to screen for *P. aeruginosa* mutants with reduced virulence and to analyse the complex interactions between this bacterium and the innate host defence response [72,73].

Other pathogens that can be studied using *Drosophila* as a model host are human parasites transmitted by insects. Interestingly, *Plasmodium gallinaceum* ookinetes injected into *Drosophila* develop into infectious sporozoites, indicating that this parasitic step can be reproduced. The use of genetic screens in *Drosophila* may thus shed light on the host requirements for *Plasmodium* development and survival [74•].

Conclusions

Our knowledge of the *Drosophila* immune response has been greatly enhanced in the past year. Some aspects, such as the function of the Toll and Imd pathways in the induction of antimicrobial peptides, have been partially clarified. However, the molecular mechanisms of other critical cellular and humoral immune responses, including phagocytosis, encapsulation, melanization and coagulation, remain unknown. Two of the most important areas for research concern the mechanisms by which pathogens are recognised and how recognition leads to the activation of the immune response. The potential use of genetic and molecular analyses in *Drosophila*, combined with the fly's complete genome sequence and with new techniques such as microarray analysis, suggests that more aspects of the immune response will be unravelled. In addition, the development of alternative methods of infection that use natural *Drosophila* pathogens offers the possibility of studying the immune response under the conditions of a real infection. Finally, completion of the genome sequencing of other insect species, such as *B. mori* and *A. gambiae*, will enable interesting comparative studies and may improve our understanding of how insects resist microbial infections.

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