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DROSOPHILA: The Genetics of Innate Immune Recognition and Response

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■ **Abstract** Because of the evolutionary conservation of innate mechanisms of host defense, *Drosophila* has emerged as an ideal animal in which to study the genetic control of immune recognition and responses. The discovery that the Toll pathway is required for defense against fungal infection in *Drosophila* was pivotal in studies of both mammalian and *Drosophila* immunity. Subsequent genetic screens in *Drosophila* to isolate additional mutants unable to induce humoral responses to infection have identified and ordered the function of components of two signaling cascades, the Toll and Imd pathways, that activate responses to infection. *Drosophila* blood cells also contribute to host defense through phagocytosis and signaling, and may carry out a form of self-nonself recognition that is independent of microbial pattern recognition. Recent work suggests that *Drosophila* will be a useful model for dissecting virulence mechanisms of several medically important pathogens.

INTRODUCTION

In 1989, Janeway proposed that innate immune mechanisms, those that rely on detection of microbes by germline encoded receptors, are ancient and essential for earliest detection of and defense against infection in mammals (1). In 1996, Lemaitre and coworkers demonstrated that the Toll receptor, previously known for its essential role during *Drosophila* embryonic development, is required for antifungal defense in *Drosophila* (2). This finding stimulated the identification of the mammalian Toll-like receptors (TLRs) and the demonstration of their importance in mammalian innate immunity. We now know that mice that lack TLRs are susceptible to infection and are impaired in the ability to activate adaptive immune mechanisms, supporting Janeway's predictions (3–5).

Well before the power of *Drosophila* genetics was harnessed to study regulation of immune responses, insects were already known to have sophisticated immune systems, involving phagocytic blood cells, serine proteolytic cascades, and inducible humoral responses, thanks to decades of biochemical work with larger insects. In particular, insects induce a number of antimicrobial peptides

upon immune challenge that are effective against Gram-negative or Gram-positive bacteria or fungi. Following the discovery of the immune function of *Drosophila* Toll, genetic screens were designed to isolate *Drosophila* mutants that could not induce particular antimicrobial peptides in response to infection.

Genetic methods have identified about two dozen genes required for induction of the antimicrobial peptides. Many of these genes encode proteins in two signaling pathways that control the activation of NF-κB-like factors in response to infection, the Toll and Imd pathways, both named for the first gene to be discovered in the pathway. Immune signaling through Toll leads to the activation of two NF-κB factors, Dif and Dorsal. Activation of the Imd signaling pathway culminates in the activation and nuclear translocation of the third *Drosophila* NF-κB-like factor, Relish.

The early finding that *Toll* mutants are impaired in survival to fungal infection and *imd* mutants impaired in antibacterial responses suggested that distinct pathways are used to detect and induce responses against bacteria and fungi. However, we now know that survival to Gram-positive bacterial infection also requires the Toll pathway. In addition to its importance in activation of antifungal responses, Toll is a central regulator of multiple aspects of *Drosophila* immunity, including resistance to bacterial infection, blood cell activation, and regulation of a melanization cascade.

Genetic screens have also identified two peptidoglycan recognition proteins (PGRPs) that bind bacterial components directly. One recognizes Gram-negative bacteria and activates the Imd pathway, and the other detects Gram-positive bacteria and triggers Toll signaling. These findings fulfil Janeway's prediction that in innate immunity, pattern recognition receptors (PRRs) would recognize conserved molecular features of microbes, or pathogen-associated molecular patterns (PAMPs). What was not foreseen in 1989, and what *Drosophila* has revealed, is that innate immune systems can discriminate among PAMPs that are characteristic of different microbial classes and activate the most appropriate defenses. Still under intense scrutiny in *Drosophila* are the mechanisms linking detection of microbes to signaling through the Imd and Toll pathways.

Other aspects of the *Drosophila* immune response are not as well understood as the signaling pathways that lead to the humoral responses, but are ripe for genetic dissection. Blood cells are activated in response to infection, but our understanding of the mechanisms and consequences of blood cell activation are fragmentary. Serine protease cascades, which also activate several aspects of the mammalian immune response (6, 7), are required for activation of Toll signaling and the melanization response in *Drosophila*.

Drosophila can also activate immune responses in the absence of microbial PAMPs—both in response to infestation with parasites, and under autoimmune conditions generated by a variety of mutations. Although the mechanisms of immune activation under these circumstances are not known, insect blood cells are able to discriminate between self and nonself, and both aberrant basement membrane patterns and endogenous DNA may be immunostimulatory.

Drosophila has recently emerged as a suitable model organism to investigate virulence mechanisms of a wide range of medically important pathogens, including *Pseudomonas, Serratia, Mycobacteria,* and malaria parasites. Many of the bacterial virulence mechanisms that are essential to establish infection in mammals also contribute to pathogenesis in flies, including type three secretion systems (TTSS) and ability to proliferate inside macrophages.

Major questions in innate immunity concern how germline encoded receptors distinguish self from nonself and discriminate among various types of foreign invaders in order to activate the most effective response. The striking commonalities between *Drosophila* and mammalian innate immunity, including Toll-NF-κB signaling, phagocytosis, serine protease cascades, PGRPs, and autoimmune defects suggest that in the years to come studies in *Drosophila* will continue to shed light on immune mechanisms that are also important in humans.

OVERVIEW OF THE *DROSOPHILA* IMMUNE RESPONSE

A Diversity of Infectious Threats

Because *Drosophila* is not an agricultural pest, there is not a long history of entomological study of their pathogens. Much of our knowledge of *Drosophila* immunity thus concerns the responses to microbes that are not normally pathogenic and do not infect wild-type *Drosophila* unless directly injected. Studying immune responses to these opportunistic infections may be particularly relevant to mammalian immunity, as generalized immune defenses tend to be more evolutionarily conserved than ones specific to individual virulent pathogens. Nevertheless, some microorganisms and parasites are known that can naturally infect *Drosophila*, permitting finer analysis of immune mechanisms without the complications that a wound can introduce (8–10).

This review primarily addresses *Drosophila* immunity to extracellular bacteria and fungi, as well as recognition of parasites, but a brief survey of a wider range of pathogens is presented.

BACTERIA *Drosophila* are very adept at eliminating invading bacteria: Larvae injected with 5000 *E. coli* CFU clear the infection within 6 h (11). Two Gram-negative bacteria are known that can naturally infect larvae through the gut (9, 12). Otherwise, *Drosophila* antibacterial responses are assessed in the lab by directly injecting Gram-negative and Gram-positive bacteria. Neither of two intracellular bacterial types studied in *Drosophila*, *Mycobacteria*, or the obligate intracellular rickettsial symbiont *Wolbachia*, elicits a humoral antibacterial response (13, 14). Specific immune defenses against intracellular pathogens are not known in *Drosophila*.

FUNGI Several fungal species, including *Beauveria bassiana*, can penetrate the cuticle of *Drosophila* and establish a lethal infection. Other fungi can be injected, and are lethal only in immunocompromised mutants (2, 8).

PARASITES Parasitoid wasps that lay their eggs in fly larvae represent a significant threat to wild *Drosophila*. In a successful infestation, the wasp egg hatches, the larva eats the fly pupa from inside, and an adult wasp eventually emerges (10, 15). The host protective response includes the encapsulation of the wasp egg by specialized lamellocyte blood cells (see below). Specific genetic loci in *Drosophila* correlate with ability to resist wasp infestation, although the genes have not yet been identified (16).

A flagellate protozoan can induce systemic antimicrobial expression from the fly gut, and kills *Drosophila* when injected (17).

VIRUSES Several viruses that can infect *Drosophila* have been characterized, including rhabdoviruses, picornaviruses, baculoviruses, retroviruses, and birnaviruses (18–23). General insect antiviral strategies are not understood, although one *Drosophila* gene required for resistance to a rhabdovirus encodes a protein that may interfere with virus replication (24).

Overview of Innate Immune Responses

There is no evidence in *Drosophila* or other insects for an adaptive immune system like that of mammals: specific antisera are not produced, and no sign of somatic gene rearrangement or a system resembling MHC antigen presentation has been found (25). *Drosophila* and most other invertebrates are thought to rely exclusively on innate immune mechanisms.

Drosophila has an open circulatory system that disseminates the mediators and effectors of immune responses, most notably the blood cells and the antimicrobial peptides. The blood, also called the hemolymph, circulates in the extracellular space, or hemocoel, which is lined with a basement membrane (26). The fat body, an analog of the mammalian liver, is an extensive monolayer sheet of cells and is the source of most of the antimicrobial peptides produced in response to systemic infection (27, 28).

ANTIMICROBIAL PEPTIDES Within hours of infection, transcription of a battery of antimicrobial peptides is induced in the fat body and the peptides are secreted into the blood. Insect antimicrobial peptides were originally isolated from larger insects based on their activities against different types of microbes and are active against fungi (Drosomycin, Metchnikowin, Cecropin), Gram-negative bacteria (Attacin, Cecropin, Diptericin, Drosocin), or Gram-positive bacteria (Defensin, Metchnikowin). Many of the peptides work by disrupting bacterial membranes (29, 30). Mutants impaired in the Imd and Toll signaling pathways that induce the antimicrobial peptide genes are severely immunocompromised (2). The ability of these mutants to resist infection can be rescued by transgenic expression of the appropriate peptides, attesting to the importance of the antimicrobial peptides in fighting infection (31).

CELLULAR RESPONSES *Drosophila* also relies on blood cells to protect against infection. Plasmatocytes, phagocytic macrophage-like cells, comprise about 90% of the blood cell population (26, 32). Crystal cells are a source of enzymes for the melanization reaction (see below) (33). Lamellocytes are extremely flattened cells that differentiate in response to certain immune challenges, and encapsulate large invaders such as parasite eggs (26, 32). The plasmatocytes may have functionally distinct subgroups. Some are sessile while others circulate, and some are more highly phagocytic than others (32). Several genes are differentially expressed among plasmatocytes, and efforts are under way to generate monoclonal antibodies that can discriminate among *Drosophila* blood cells (34, 35).

Phagocytosis is a vital contribution of blood cells to immunity; most bacteria injected into a fly are taken up by the blood cells within minutes (26, 36). Blood cells are required for signaling to the fat body under some infection conditions (9, 37). They also accumulate at wound sites and help form clots (33, 38). The importance of blood cells in fighting infection is shown by sensitization to infection seen when phagocytosis is blocked or in mutants that lack blood cells (36, 39).

Although it is beyond the scope of this review, there is significant homology between *Drosophila* and mammalian hematopoieisis. Both require the function of genes in the GATA, NF-κB, Notch, Runt/AML1, JAK-STAT, Ras, and VEGF families and pathways (40–48).

MELANIZATION The deposition of melanin is a rapid, highly localized defense triggered by wounding and the presence of foreign invaders. Melanization contributes to wound clotting and encapsulation of wasp eggs, and produces toxic intermediates including reactive oxygen species. Phenoloxidase, which catalyzes melanin production, is maintained as an inactive zymogen and is activated by a serine protease cascade (49). A mutant lacking hemolymph phenoloxidase is sensitized to infection and is vulnerable to death from wounds (38, 39).

BARRIER EPITHELIA All the surface epithelia of *Drosophila* that contact the environment, including the exterior, the gut, and the tracheae, induce antimicrobial gene expression upon contact with microbes (50–52). The Imd pathway regulates the induction of all peptides in the epithelia, including antifungal peptides (50–52). In mammals, antimicrobial peptides also play key roles in epithelial defenses against infection (53, 54).

The Question of Specificity

Can innate immune systems tailor responses to the type of immune challenge? Some aspects of the *Drosophila* humoral response are highly specific. For example, fungal infection specifically induces *Metchnikowin* and *Drosomycin*, the two antifungal peptides (8). Infection with Gram-negative bacteria, on the other hand, induces many antimicrobial peptides, even the antifungal *Drosomycin* (9, 37). However, some specificity is apparent because *Drosomycin* is induced only transiently by Gram-negative bacterial infection, whereas the expression of antibacterial

peptides is sustained (8, 55). Selective activation by different microbes of either the Imd or the Toll pathway has been proposed to account for the specificity of immune response in *Drosophila*. However, there is evidence that the system is more complex than this, which is discussed below.

Although there is no evidence in insects for a system of adaptive immunity like that in mammals, the possibility of specific immunological memory has not been excluded. Indeed, the recent finding that some arthropods are able to transfer specific immunity to their offspring suggests that a system of inducible immunological memory could also exist in insects (55a). Recognition and memory of evolved pathogens likely involves molecules that remain to be characterized.

THE IMD AND TOLL PATHWAYS CONTROL THE HUMORAL RESPONSES

Signaling through the Imd and Toll pathways results in the translocation of distinct NF-κB factors to the fat body nucleus and accounts for most of the transcriptional induction of genes in response to fungal and bacterial infection (2, 56–59) (Figure 1).

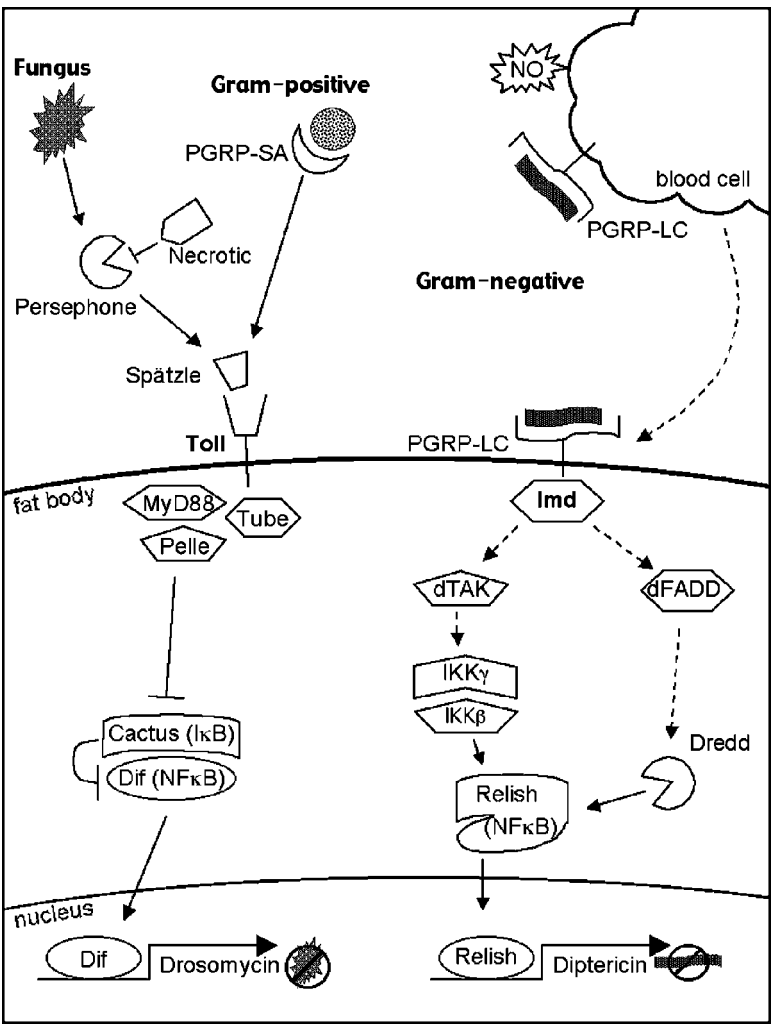
Toll Pathway

Adult *Drosophila* mutants lacking the function of Toll pathway elements are unable to induce *Drosomycin* in response to fungi and are susceptible to fungal infection,

Figure 1 Imd and Toll signaling pathways activate humoral antimicrobial defenses in the *Drosophila* fat body. Toll signaling is activated by fungal and Gram-positive invaders by different mechanisms. By an unknown mechanism, fungi trigger a cascade involving the serine protease Persephone, which results in the proteolytic activation of Spätzle, a ligand for Toll. Gram-positive invaders are recognized by an independent process that requires a circulating peptidoglycan recognition protein. Toll signaling culminates in the translocation of the NF-κB factor Dif to the nucleus where it activates transcription of the antifungal *Drosomycin* and other genes. Gram-negative bacteria are recognized by a transmembrane peptidoglycan receptor, PGRP-LC. In at least some cases (see text), blood cells are required for induction of defenses against Gram-negative bacteria, suggesting that the blood cells may signal to the fat body. Nitric oxide (NO) is implicated in blood cell activation of antibacterial defenses in the fat body, although its specific role is unknown. PGRP is upstream of the Imd pathway that culminates in the phosphorylation and cleavage of the Relish NF-κB factor, which enters the nucleus and activates many genes including the antibacterial *Diptericin*. Overexpression of Imd and several other constituents of the Imd pathway causes constitutive *Diptericin* expression. Genetic epistasis between overexpressing transgenes and mutations in other genes has tentatively ordered the pathway as shown. The dotted line indicates relationships suggested by overexpression experiments or physical associations.

yet they are able to induce *Diptericin* normally and resist most Gram-negative bacterial infections (2, 59–62). Toll signaling was thus originally considered an antifungal pathway. Interestingly, however, mutants lacking Toll pathway elements are also susceptible to Gram-positive infection (59, 63) (see Figure 1).

Toll, first discovered in *Drosophila*, is a transmembrane protein with extracellular leucine-rich repeats and an intracellular signaling domain similar to that of the Interleukin-1 receptor (64). The ligand for *Drosophila* Toll is a circulating endogenous protein, Spätzle, which is proteolytically activated by a serine protease cascade in response to infection (65, 66). Ligand binding to Toll activates a cytoplasmic cascade involving dMyD88, the IRAK-like kinase Pelle, and the adaptor



protein Tube. This results in the degradation of the IκB-like Cactus, permitting the nuclear translocation of the NF-κB-like proteins Dif and Dorsal (2, 56, 57, 62, 67–69). Most of the Toll pathway mutants were originally isolated for their maternal effect embryonic patterning defects; the mutants are viable, but females produce abnormally patterned embryos (70). *Dif* and *dMyD88* mutants were isolated by reverse genetic approaches (56, 60–62).

Although *Drosophila* has 9 Toll receptors, they are not believed to provide specificity by recognizing different PAMPs as they are in mammals. Toll does not directly bind microbial components. None of the other *Drosophila* Tolls have been identified in genetic screens for immunodeficient mutants or are upregulated by immune challenge (71). An early report that 18Wheeler (the second *Drosophila* Toll) had specific antibacterial defects has not been confirmed (72, 73). Several of the other *Drosophila* Tolls have morphogenetic and neural functions (74, 75). Sequence comparisons suggest that the last common ancestor of mammals and invertebrates may have had only one or a few Toll receptors that subsequently duplicated and diverged under different selection pressures in the two lineages (76). Some of the other *Drosophila* Tolls may have immune functions, but assessment of their contributions awaits loss-of-function analysis (Table 1).

Imd Pathway

The Imd signaling pathway mediates the induction of *Diptericin* and other antibacterial peptide genes in the fat body in response to Gram-negative bacterial infection, and bears some resemblance to the mammalian TNF-α pathway (Figure 1) (77–79). Although Imd itself was discovered serendipitously, most Imd pathway components were identified in genetic screens for mutants unable to induce reporters of *Diptericin* expression in response to bacterial challenge, or to survive infection (36, 80–83).

The Imd signaling pathway culminates in the activation of the NF-κB-family member Relish. Relish, like mammalian p100 and p105, is a compound protein with an N-terminal Rel domain and C-terminal ankyrin repeat domain and is proteolytically processed in response to upstream signals to generate an N-terminal Rel protein that enters the nucleus and activates transcription of target genes including *Diptericin* (58, 84). Proteolytic activation of Relish differs from that of p100 and p105 in that it is proteasome-independent and mediated directly by the caspase Dredd (58, 85). Relish activation also requires phosphorylation by an IκB kinase (IKK) complex (11, 83, 86). The genes encoding dTAK (a MAPKKK) and the adaptor protein dFADD are also required for induction of antibacterial peptide genes (87–90). All the Imd pathway mutants are unable to induce *Diptericin* and are susceptible to bacterial infection (2, 36, 82–84, 88, 89, 91, 92), whereas in vivo overexpression of Imd and other pathway elements causes constitutive *Diptericin* expression (87, 88, 91). Imd pathway mutants are often able to resist Gram-positive infection almost as well as wild-type (59, 63, 92).

Negative regulators in the Imd pathway cannot be recovered in screens for immunodeficient mutants, but a screen for mutants that overexpress *Diptericin*

TABLE 1 Proteins that may activate immune responses in response to microbial invasion. The roles of these proteins have not been confirmed by mutant analysis

Gene(s)	Type of protein encoded	Evidence	References
PGRP-LE	PRR	In vivo overexpression induces constitutive *Diptericin* and melanization	(117)
dSR-C1	Scavenger receptor	RNAi in S2 blood cell line impairs ability to bind and phagocytose bacteria	(118)
GNBPs	Secreted; similar to bacterial β-1,3 glucanases and CD14 (3 in *Drosophila*)	Binds LPS, β-1,3 glucan; overexpression in S2 cells increases ability to induce antimicrobial peptides	(116, 119)
TEPs	Thiolester-containing; complement factor C3-like; opsonin?	Upregulated upon infection; RNAi of mosquito homolog impairs Gram-negative phagocytosis	(99, 120)
Masquerade	"Inactive" serine protease (null mutations lethal)	Upregulated upon Gram-positive, fungal infection; crayfish homolog is an opsonin	(55, 121)
dToll5	Toll-like receptor	Chimeric constitutively active protein with dToll5 cytoplasmic domain activates Drosomycin in S2 cells; coreceptor with Toll?	(122, 123)
dToll9	Toll-like receptor	Wild-type protein constitutively activates Drosomycin in S2 cells in absence of infection; through canonical Toll pathway	(124)

identified a ubiquitin ligase complex which targets Relish for destruction in the absence of infection (93).

Other Signaling Pathways

The JAK-STAT and JNK pathways are important in the mammalian immune response (94, 95) and also signal during the *Drosophila* immune response. LPS stimulation of *Drosophila* blood cells activates JNK within minutes (96). Microarray analysis of RNAi-treated blood cells exposed to LPS indicates that the JNK pathway controls the rapid upregulation of cytoskeletal genes in response to infection (90). Although null JNK pathway mutants die as embryos due to a requirement for this pathway for early developmental events including epithelial fusion (97), a hypomorphic allele of DFos, a transcriptional effector of JNK signaling, impairs wound healing (38).

JAK-STAT signaling in *Drosophila* is required for the induction in the fat body of a number of genes in response to infection, including the stress-induced gene *totA*, as well as *Tep1*, which encodes a thiolester-containing protein that may be an opsonin (see Table 1) (90, 98, 99). Upd3 is a cytokine-like protein produced by the blood cells upon infection, and it activates transcription of *totA* in the fat body by signalling through the receptor Domeless and the JAK-STAT pathway (98). In general, dependence on JAK-STAT signaling correlates with delayed, transient induction following immune challenge (90). In addition, constitutive JAK signaling hyperactivates the blood cells (see below).

PATTERN RECOGNITION AND ACTIVATION OF IMD AND TOLL SIGNALING

The Imd and Toll pathways can be activated through binding by specific PGRPs of Gram-negative or Gram-positive bacterial molecules, respectively. It is not yet clear how fungal recognition activates Toll signaling, nor have the functions of other candidate PRRs been defined.

Recognition of Microbes

PGRPs were first identified in moths as infection-induced proteins that bind peptidoglycan, triggering the proteolytic melanization cascade (100, 101). The *Drosophila* genome encodes at least 13 PGRPs, some with multiple splice-forms, and the human genome encodes 4 that also have splice variants (102–104). PGRPs share a 160 amino acid peptidoglycan recognition domain, and both mammals and *Drosophila* have genes that encode secreted (S) and transmembrane (L) forms (101). In *Drosophila*, a secreted PGRP is required for survival to Gram-positive bacteria, whereas mutants lacking the function of a transmembrane PGRP are susceptible to Gram-negative bacterial infection (92, 105–107). In addition, at least one of the *Drosophila* secreted PGRPs scavenges and degrades peptidoglycan (108). A hydrophobic groove shared by both secreted and transmembrane PGRPs may function to bring downstream effectors into proximity, promoting signaling (109), while the cytoplasmic tails of transmembrane PGRPs may also contribute to downstream signaling.

PGRP-LC AND GRAM-NEGATIVE DETECTION Mutants in PGRP-LC fail to induce the antibacterial peptides and are susceptible to Gram-negative but not Gram-positive infection (92, 106, 107). The transmembrane PGRP-LC has two major splice variants that share common transmembrane and cytoplasmic domains but have very different extracellular peptidoglycan recognition domains (103, 106). PGRP-LC acts upstream of the Imd pathway: Mutants are unable to proteolytically activate Relish during infection, and constitutive expression of *Diptericin* caused by PGRP-LC overexpression requires wild-type Imd function (92, 106).

It was surprising to find a PGRP implicated in the recognition of Gram-negative bacteria because Gram-negative peptidoglycan (PG) is in the inner cell wall layer, which is covered by the outer membrane. In contrast, the PG of Gram-positive bacteria is much more accessible on the cell wall surface. Lipopolysaccharide (LPS), on the other hand, is an abundant constituent of the Gram-negative outer membrane, and is an extremely immunogenic molecule in mammals (110). Because LPS is not found in Gram-positive bacteria, it was expected to be the key to the discrimination between Gram-negative and Gram-positive bacteria by *Drosophila*.

However, *Drosophila* is able to discriminate between Gram-negative and Gram-positive PG, which differ in a single amino acid, whereas LPS is not a potent inducer of the humoral response in vivo (111, 112). The PGRPs play an essential role in the recognition of and disrimination between Gram-negative and Gram-positive bacteria. Gram-negative PG, but not Gram-positive PG, is a potent inducer of *Diptericin* in flies and cultured blood cells, and requires PGRP-LC function (103, 112, 113). PGRP-LC was also identified in a blood cell RNAi screen as a protein involved in Gram-negative, but not Gram-positive, binding and phagocytosis (107).

PGRP-SA AND GRAM-POSITIVE DETECTION A mutant lacking the function of PGRP-SA was identified in a screen for mutants unable to induce *Drosomycin* following a mixed bacterial infection (105). Although *Drosomycin* is an antifungal peptide, it is induced through the Toll pathway by both fungi and Gram-positive bacteria. *PGRP-SA* mutants are specifically susceptible to Gram-positive infection; resistance to fungi and Gram-negative bacteria is normal (105). Consistent with this, *PGRP-SA* mutants cannot induce *Drosomycin* in response to Gram-positive infection or Gram-positive PG, although they induce *Drosomycin* normally in response to fungal infection. *Diptericin* is induced normally in response to Gram-negative PG or whole bacteria (105, 112).

PGRP-SA is a secreted PGRP consisting mainly in a single peptidoglycan recognition domain, and binds Gram-positive PG with high affinity (102, 105). The inability of the mutants to induce *Drosomycin* suggests that PGRP-SA may activate the Toll pathway, although this has not been tested genetically.

Some aspects of secreted PGRP function may be evolutionarily conserved. A mouse mutant lacking the function of a secreted PGRP is also vulnerable to Gram-positive infection (114).

OTHER POSSIBLE PATTERN RECOGNITION PROTEINS Other *Drosophila* proteins may have roles in recognizing microbial invaders, but because no mutants have yet been isolated, their requirements in immune function have not been directly tested. Some of these are listed in Table 1. Currently, there are no genetic data concerning host molecules involved in detection of fungi, although fungal β-1,3-D-glucans induce antimicrobial gene expression in a *Drosophila* blood cell line, and a β-1,3-glucan-binding protein contributes to this response (115, 116).

From Recognition to Fat Body Signaling

A major focus of current research is how pattern recognition events involving PGRPs or other host receptors leads to activation of the fat body NF-κB signaling pathways that induce the antimicrobial peptides. The importance of a circulating PGRP for Gram-positive detection and a transmembrane PGRP for Gram-negative detection suggests that the fly immune system detects these two classes of bacteria rather differently. Gram-positive detection has more in common with fungal pathways in the dependence on the Toll pathway, and possibly also on serine proteolytic cascades.

ACTIVATION OF THE IMD PATHWAY The fat body is the source of the majority of the antimicrobial peptides produced in a systemic immune response. Relish translocation can be visualized here, and Imd signaling presumably takes place in fat body cells. However, the site of microbial detection by PGRP-LC may be elsewhere. Whereas constitutive fat body *Diptericin* expression can be induced by overexpressing PGRP-LC in the adult fat body, endogenous larval PGRP-LC expression is higher in the blood cells than the fat body (92, 102, 106). Consistent with this, in larvae, blood cells are required for the induction of *Diptericin* in the fat body in response to a Gram-negative gut infection (9, 37). These data suggest that blood cells might detect Gram-negative microbes and signal this information to the fat body. Blood cells are not required for induction of fat body *Diptericin* when Gram-negative bacteria are introduced through a wound (39), which may be the result of a signal generated at the site of injury.

NO is implicated in the blood cell–dependent induction of fat body *Diptericin*. Exogenous NO induces *Diptericin*, and a pharmacological inhibitor of nitric oxide synthase (NOS) prevents *Diptericin* induction in response to a Gram-negative infection (37, 125). The response to NO requires Imd, suggesting that NO acts upstream of the Imd pathway. However, NO is unlikely to be the signal from blood cells to the fat body, as exogenous NO does not stimulate *Diptericin* induction in a mutant that lacks blood cells (37). NO appears to be important in some step in blood cells downstream of microbial recognition and upstream of a signal that is relayed to the fat body, possibly in blood cell activation.

ACTIVATION OF THE TOLL PATHWAY In the *Drosophila* embryo, the Toll pathway is triggered by a cascade of four serine proteases that proteolytically activate the endogenous ligand Spätzle (126). Although Spätzle is also required for immune responses, the embryonic proteases are not (2). Necrotic is a serine protease inhibitor (serpin) that prevents constitutive activation of Toll-dependent immune responses in the absence of immune challenge, suggesting that a distinct serine proteolytic cascade activates Spätzle in immune responses (65).

Persephone, an immune response serine protease, was identified in a screen for mutations that suppressed the ability of *necrotic* mutants to constitutively activate Toll signaling. *persephone* mutants are susceptible to fungal infection and unable

to activate the Toll pathway in response to fungi (127). It is unclear how fungal infection leads to Persephone activation, but the possession of a prodomain suggests that Persephone may be activated by another serine protease (127). Necrotic may directly inhibit Persephone; fungal infection is likely to shift the balance between protease and serpin activities (127, 128). It is also not known whether Persephone directly activates Spätzle, or whether there are additional intervening proteases.

Activation of the Toll pathway by Gram-positive bacteria does not require Persephone, suggesting that fungi and Gram-positive bacteria activate Toll signaling by distinct mechanisms (127). Because Spätzle is required for responses to some Gram-positive bacteria, PGRP-SA can likely activate a distinct serine protease cascade that leads to Spätzle cleavage (59, 63, 105).

Do Imd and Toll Really Provide Specificity of Response?

The model that selective activation of the Imd or Toll pathways confers specificity of response against Gram-negative bacteria or against Gram-positive bacteria and fungi, respectively, is an oversimplification. For example, Imd Toll pathway double mutants are more susceptible than single Imd pathway mutants to *E. coli* infection, and Toll pathway mutants are susceptible to *Pseudomonas* infection, arguing that Toll is important for resistance to Gram-negative infection (59, 129). On the other hand, Imd signaling is important for resistance to some Gram-positive bacteria such as *Micrococcus luteus* (59, 82).

Another difficulty with the model that the Imd and Toll pathways confer specificity of response is the sharing of the Toll pathway by fungi and Gram-positive bacteria. Despite the use of distinct mechanisms for detecting fungi and Gram-positive bacteria, the signals apparently converge at Spätzle and Toll. In fact, some responses to Gram-positive bacteria and fungi are remarkably similar: Both types of infection trigger the massive induction of Masquerade, a serine protease-like protein, whereas Gram-negative infection does not (55). Another similarity between fungal and Gram-positive immune induction is the apparent reliance on circulating, rather than cell-associated detection mechanisms. Why would an immune system have one pathway dedicated to Gram-negative defense and another for responses to microorganisms as disparate as fungi and Gram-positive bacteria?

It could be that the Toll pathway is the ancestral immune induction pathway in insects (Figure 2), and that a blood cell-mediated system to recognize Gram-negative bacteria evolved later, triggering a distinct pathway. The Imd pathway may also have an apoptotic role, suggesting that its immune function may have been co-opted secondarily (91). There may be features of Gram-negative bacteria that selected for an additional immune detection and induction mechanism, such as a higher growth rate or concealment of PG beneath the outer membrane. Indeed, a genome-wide microarray analysis of the kinetics of induction of immune-responsive genes in wild-type and mutant conditions found a correlation between Imd regulation of early activated genes and Toll regulation of genes with a more delayed response (90). Specialization of Imd signaling for rapid responses would be

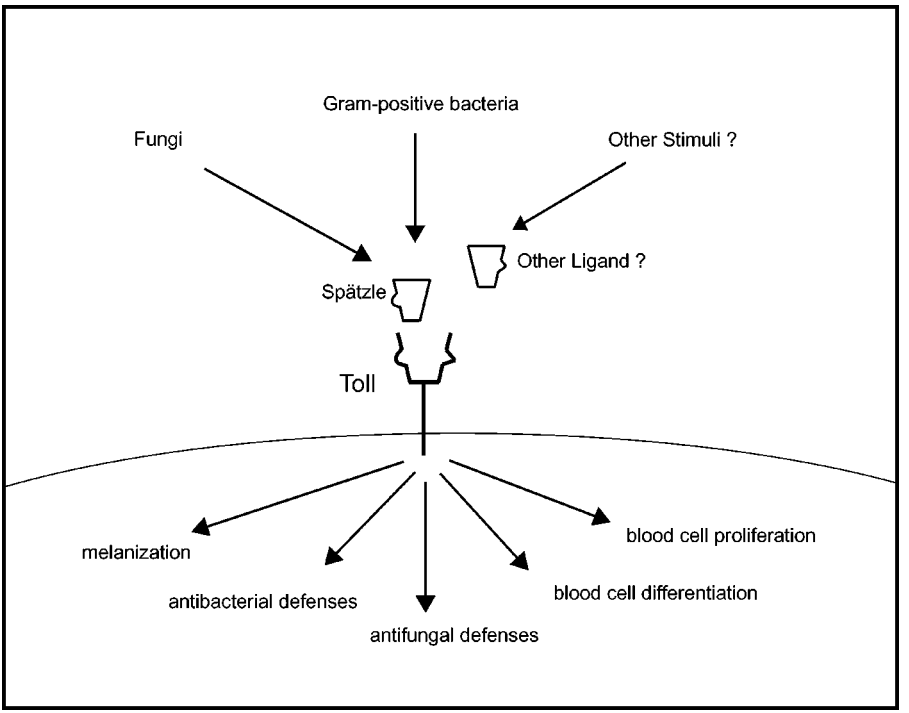


Figure 2 Toll is a central regulator of the *Drosophila* immune response. In agreement with the idea that Toll may represent the ancestral immune signaling pathway in insects, Toll regulates many aspects of the immune response. In addition to activating antifungal defenses, Toll is also required for survival to many Gram-positive and some Gram-negative bacterial infections, for regulation of the melanization cascade and for regulation of blood cell proliferation, and is implicated in blood cell differentiation and activation. Toll may fulfill these roles in several tissues, such as the fat body and the blood cells. Spätzle is the only known ligand for Toll in *Drosophila*; however, *Toll* mutants are more impaired in the induction of antimicrobial peptides than are null *spätzle* mutants (2), suggesting that there may be additional Toll ligands.

consistent with the independence of Imd of both *Drosomycin* induction, which has slow kinetics, and survival to fungi, which are slower-growing microorganisms.

ACTIVATION OF MELANIZATION AND BLOOD CELL RESPONSES

In addition to the well-characterized antimicrobial peptide induction, infection also triggers blood cell activation and melanization, events that are less well understood in *Drosophila*.

Activation of the Prophenoloxidase Pathway

Biochemical experiments in the silkworm and crayfish revealed that a serine protease cascade regulates the activation of prophenoloxidase (PPO), which catalyzes melanin production (49, 130–132). An important regulator of the melanization response is Serpin27A (Spn27A), which specifically inhibits the PPO-activating enzyme (PPAE) (133, 134). *Spn27A* mutants exhibit sporadic melanization in the absence of immune challenge, and the entire animal becomes melanized after septic injury (133, 134).

The inhibitory effect of Spn27A on melanization appears to be overcome in two ways under conditions of immune challenge. The melanization cascade can be rapidly triggered by microbial products, by mechanisms that involve PGRPs (100, 117, 131) and that likely activate an upstream protease that leads to hyperproduction of PPAE, depleting Spn27A. An additional uncharacterized inhibitor of Spn27A may be transcriptionally induced by Spätzle-Toll signaling (134). It appears that rapid activation of a protease cascade, triggered by pattern recognition, initiates an immediate melanization response, and that sustained activation is ensured by local Toll-mediated depletion of Spn27A.

Activation of Blood Cells

PHAGOCYTOSIS Phagocytic uptake of invaders by blood cells is a vital defense strategy in both flies and mammals (36, 135). *Drosophila* and mammalian phagocytosis are homologous actin-mediated processes (118). Critical unanswered questions in both mammalian and *Drosophila* phagocytosis include how ingested particles are trafficked and the role of phagocytosis in stimulating other immune responses (135).

The ability of a blood cell to phagocytose a particular particle is influenced by the affinity of host receptors for surface molecules on the particle. For example, a *Drosophila* scavenger receptor-like protein, dSR-C1, confers the ability to phagocytose bacteria but not yeast on a heterologous cell line (118). However, *Drosophila* blood cells are able to phagocytose abiotic particles such as polystyrene beads, so pattern recognition of microbial PAMPs is not an absolute requirement (36).

Few *Drosophila* mutants are detectably impaired in phagocytosis, possibly reflecting redundancy of mechanisms promoting particle uptake and requirements for cytoskeletal proteins for viability. An RNAi screen in cultured blood cells tested the requirements for 1000 randomly selected genes in phagocytosis of different microbes, and defined roles for proteins involved in cytoskeletal function and vesicle formation and transport (107). In addition, the removal of PGRP-LC function impaired the binding and phagocytosis of Gram-negative bacteria by approximately 30% (107). Null *PGRP-LC* mutants are not detectably impaired in phagocytosis, suggesting either that a 30% reduction in phagocytic ability is not detectable in vivo or that additional mechanisms compensate in vivo for the loss of this receptor (106). Several *Drosophila* proteins have been proposed to bind microbes and promote their uptake by blood cells (Table 1).

OTHER ASPECTS OF BLOOD CELL ACTIVATION Wasp parasitization triggers blood cell proliferation, the differentiation of the lamellocyte blood cell type, and an encapsulation response (see below) (32, 35, 136). Although the regulation of these dramatic events is not understood, they are mimicked in mutants with hyperactivated Toll and JAK-STAT signaling (42, 137, 138) (Figure 3). Toll also regulates steady-state hemocyte numbers: There are fewer blood cells in loss-of-function mutants (42). In addition, blood cells accumulate at wound sites (38); because blood cells with overactivated Toll signaling also aggregate, Toll could also be involved in this type of activation (42). In short, Toll signaling is implicated in several aspects of blood cell activation in *Drosophila*, but the regulation of blood cell activation in response to actual immune challenge is not understood.

IMMUNE RESPONSES IN THE ABSENCE OF MICROBIAL PATHOGENS

PAMPs are most clearly defined for bacteria and fungi, microorganisms from entirely different kingdoms than animals. However, like mammals, *Drosophila* is able to mount immune responses in the absence of PAMPs, for example during parasite infestation and under autoimmune conditions. Deviations from the normal basement membrane pattern as well as presence of endogenous DNA in the blood are both associated with immune activation.

Basement Membrane

PARASITES A parasitic wasp egg in a *Drosophila* larva triggers an encapsulation immune response designed to seal off and kill the wasp embryo before it can hatch and kill the larva. Circulating plasmatocytes appear to recognize the wasp as foreign, and attach to it. Lamellocytes then adhere in layers, and the entire capsule is melanized (32, 139). This protective response has some similarities to granuloma formation by mammalian macrophages and T cells (140). Because wasps are also insects, they are not expected to have obligate molecular signatures, or PAMPs, that allow them to be recognized as invaders. How, then, are wasp eggs recognized as foreign?

Transplantation experiments suggest that *Drosophila* blood cells may recognize the absence of endogenous or presence of foreign basement membrane on invaders as nonself. *Drosophila* does not encapsulate fat bodies of within-species transplants, but fat bodies transplanted from other species are encapsulated (141).

MELANOTIC CAPSULES In some mutants, *Drosophila* blood cells aberrantly encapsulate the fly's own tissue, representing a kind of autoimmune defect (Figure 3). The resulting melanotic capsules resemble encapsulated parasitoid eggs, with layers of melanized lamellocytes. Although melanotic capsules can appear in mutants with constitutively activated blood cells (Figure 3), they also occur in mutants in which damaged or aberrant tissues trigger an immune response (142).

Several melanotic capsule mutants show disruption of the basement membrane surrounding various tissues and the subsequent encapsulation of the exposed tissues. For example, in *Tu-Sz[ts]* mutants, disruption of the basement membrane over part of the fat body precedes the adhesion of lamellocytes and formation of melanotic capsules there (26). Transplanted *Drosophila* fat bodies whose basement membranes have been damaged are encapsulated, whereas intact fat bodies are not, supporting the notion that disruptions to the endogenous basement membrane can trigger immune responses (141).

BLOOD CELLS AND BASEMENT MEMBRANE The blood cells are implicated in the recognition of basement membrane abnormalities in parasite infestation and autoimmune activation. The notion of blood cells constantly surveying the basement membrane lining the hemocoel is consistent with their roles in basement membrane secretion, repair, and degradation (26, 33, 143). Blood cells seem to be able to distinguish the basement membrane of healthy self from that of damaged self, as well as absence of basement membrane on abiotic material, and possibly presence of foreign basement membrane on parasites. The basement membrane is a proteoglycan-rich matrix, and experiments in other insects, in which beads coated with different materials were transplanted, suggest that the self characteristics may be related to carbohydrate composition (33, 144).

Endogenous DNA

In addition to those with basement membrane defects, some other mutants with melanotic capsules have defects in apoptosis. Mutants lacking the function of the fly proapoptotic homologs of Ced-3 caspase and Ced-4/Apaf-1 develop melanotic capsules (145, 146). *Drosophila* blood cells do normally recognize and phagocytose apoptotic cells through a CD36-like receptor called Croquemort (147). It is possible that cells failing to undergo proper apoptosis are recognized as abnormal, triggering encapsulation. Alternatively, cells undergoing aberrant apoptosis might release immunostimulatory molecules. Ced-3 protein is able to fragment DNA; perhaps DNA in the blood of Ced-3 caspase mutants triggers an immune response resulting in encapsulation of self tissue (145).

Fly mutants lacking the function of two other DNAses required for the degradation of apoptotic cell DNA show constitutive expression of *Diptericin* (148). This further suggests that endogenous DNA may stimulate the innate immune system of *Drosophila* as it does in mammals (149).

STUDYING MEDICALLY IMPORTANT PATHOGENS IN *DROSOPHILA*

Drosophila has recently emerged as a very promising system in which to study the virulence of a variety of medically important pathogens. Several microbes have been shown to infect flies with similar mechanisms to those known from mammals.

Because *Drosophila* is a genetically tractable animal with a sophisticated, blood-cell-dependent innate immune system, the possibilities for increasing our understanding of pathogenesis are tremendous.

Pseudomonas aeroginosa is a ubiquitous Gram-negative bacterium that is responsible for opportunistic infections in wounds and in immunocompromised patients. Remarkably, some of the mechanisms used by this bacterium to infect and kill the host are common to mammals, insects, nematodes, and plants (150). All 11 virulence factors required for maximal pathogenicity in mammals are also required for maximum virulence in flies (129). The TTSS, which contributes to virulence in both mammals and insects, is activated upon entry into *Drosophila* (151). This is one of the first reports of activation of the TTSS of any bacterial species in a whole animal, suggesting that *Drosophila* may be a suitable in vivo alternative to simulating combinations of signals in vitro. A screen for *P. aeroginosa* mutants impaired in *Drosophila* killing led to the identification of a gene cluster that regulates motility factors important for virulence in flies and mammals (152).

Serratia marcescens is a Gram-negative insect pathogen responsible for opportunistic infections in humans. Several *S. marcescens* mutants impaired in *Drosophila* killing are also attenuated in mammalian infection models (153).

Like *Mycobacterium tuberculosis*, *M. marinum* can proliferate inside vertebrate macrophages in which phagosome acidification has been blocked. *M. marinum* can also infect *Drosophila* blood cells by a similar mechanism, killing the fly. *M. marinum* upregulates some of the same genes in vertebrate and insect phagosomes, and at least one bacterial virulence factor contributes to pathogenesis in both systems (13).

A Gram-negative plant pathogen, *Erwinia carotovora*, that may be spread by *Drosophila*, has also provided tools for the study of mammalian disease. A novel virulence factor was identified from a genetic screen for *E. carotovora* mutants unable to infect *Drosophila* larvae. This gene is sufficient to confer ability to infect *Drosophila* not only on noninfectious *E. carotovora* strains, but also on other *Enterobacteria* such as *E. coli* and *Salmonella typhimurium* (154). These new strains of medically important bacteria that are infectious in *Drosophila* may prove to be powerful tools to investigate virulence mechanisms.

In addition to serving as lab models for medically important bacterial diseases, insects are also the natural vectors for many pathogens that infect mammals. Although malaria-causing *Plasmodia* are transmitted by mosquitoes, *Plasmodia* are able to infect *Drosophila* in the lab and progress through several steps of their complex life cycle (155). In addition, knowledge of insect immunity that *Drosophila* studies have yielded is being applied to studies of mosquito-*Plasmodium* infections with the goal of developing antimalaria strategies (156).

FUTURE DIRECTIONS

In the eight years since *Toll* and *imd* mutants were found to be differentially susceptible to fungal and bacterial infection (2), and the six years since the first genetic screen for immune defects was reported (57), genetic analysis has revealed many of

the essential aspects of these two signaling pathways. Continued forward genetic screens, as well as reverse genetic analysis of genes regulated by infection, will identify more components required for diverse aspects of the immune response. We can anticipate that future research in *Drosophila* immunity is likely to identify more novel pattern recognition mechanisms, define mechanisms of blood cell activation, illuminate interactions between NF-κB and other signaling pathways, define mechanisms that allow recognition of intracellular bacterial and viral infections, provide perspectives on autoimmunity, and define specific responses to pathogenic organisms.

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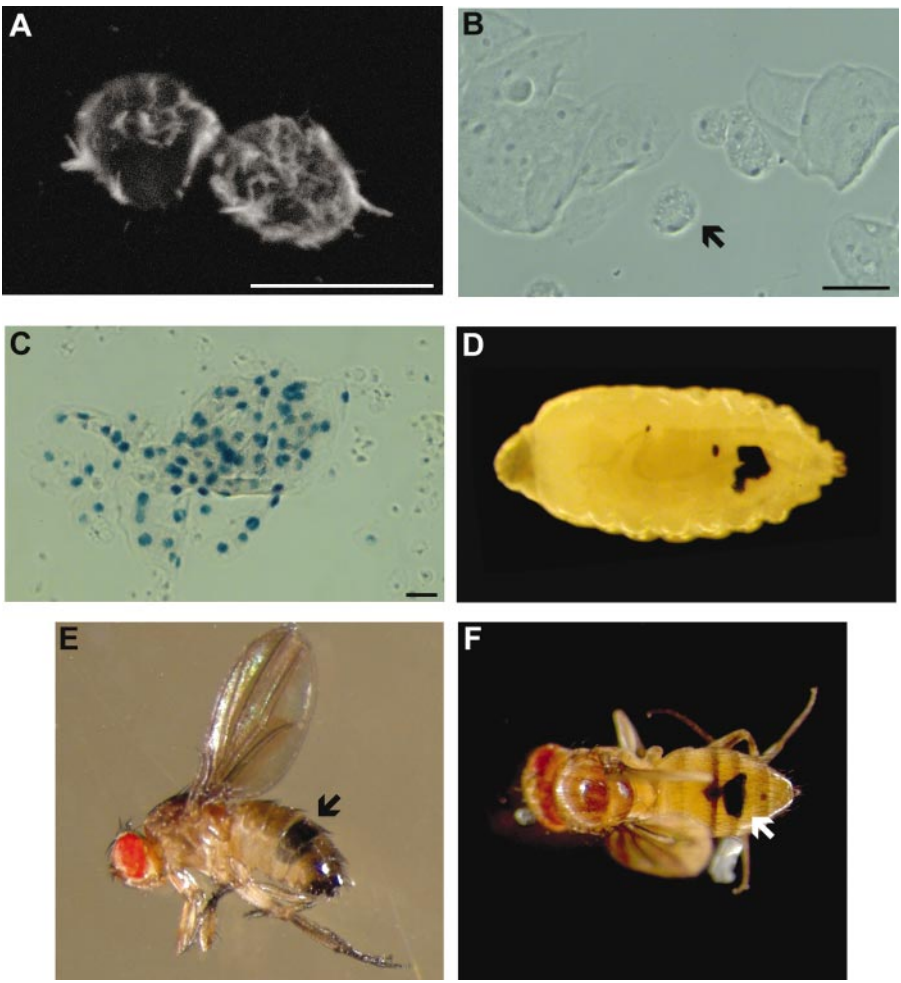

Figure 3 *Drosophila* blood cells in normal and autoimmune conditions. (*A*) Two plasmatocytes stained with phalloidin, showing filamentous actin. (*B*) Phase contrast images of two blood cells from a larva with a gain-of-function allele of Toll, *Toll*^{10b}. Constitutive Toll signaling causes the differentiation of lamellocytes, the large flat cells. The arrow indicates a plasmatocyte. (*C*) The blood cells of a *Toll*^{10b} larva carrying an enhancer trap that expresses *lacZ* in lamellocytes. Here the lamellocytes are beginning to encapsulate self-tissue, but melanization has not yet begun. Scale bars in (*A*), (*B*), and (*C*) are all 10 µm. Melanotic capsules (*arrows*) are visible through the cuticle of *Toll*^{10b} larvae (*D*), and adults (*E*) and (*F*).