The Host Defense of *Drosophila melanogaster*

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Annu. Rev. Immunol. 2007.25:697-743. Downloaded from arjournals.annualreviews.org by Swiss Academic Library Consortia on 01/22/09. For personal use only.

Annu. Rev. Immunol. 2007. 25:697–743

First published online as a Review in Advance on January 2, 2007

The *Annual Review of Immunology* is online at immunol.annualreviews.org

This article's doi:
10.1146/annurev.immunol.25.022106.141615

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0732-0582/07/0423-0697$20.00

Key Words

insect immunity, Toll, Imd, recognition, pathogens

Abstract

To combat infection, the fruit fly *Drosophila melanogaster* relies on multiple innate defense reactions, many of which are shared with higher organisms. These reactions include the use of physical barriers together with local and systemic immune responses. First, epithelia, such as those beneath the cuticle, in the alimentary tract, and in tracheae, act both as a physical barrier and local defense against pathogens by producing antimicrobial peptides and reactive oxygen species. Second, specialized hemocytes participate in phagocytosis and encapsulation of foreign intruders in the hemolymph. Finally, the fat body, a functional equivalent of the mammalian liver, produces humoral response molecules including antimicrobial peptides. Here we review our current knowledge of the molecular mechanisms underlying *Drosophila* defense reactions together with strategies evolved by pathogens to evade them.

AMP: antimicrobial peptide

RNAi: RNA interference

INTRODUCTION

Insects and microorganisms coexist within the biosphere in numerous ways. Frequently, insect larvae develop in decaying organic matter, and insect adults often serve as vectors for microorganisms causing plant and animal diseases. Thus insects have evolved sensitive mechanisms for recognition of pathogens and an array of strategies to defend themselves against attacks by bacteria, fungi, parasites, and viruses. To combat infection, the fruit fly *Drosophila melanogaster* relies on multiple innate defense reactions that are partially shared with higher organisms (1–4) (**Figure 1**). The mechanisms regulating these immune responses have been largely uncovered with the aid of genetic and molecular studies in *Drosophila*. The key role of the *Drosophila* model for studying immunity was illustrated by the initial genetic identification of signaling pathways mediating antimicrobial peptide (AMP) gene expression (5). Relatives of the Toll receptor protein, the cell surface receptor of the *Drosophila* Toll signaling pathway, also regulate innate immune responses in mammals. The conservation of a signaling pathway for the activation of antimicrobial defense responses suggests that some components of innate immunity share an ancient origin in metazoan evolution and demonstrates that *Drosophila* is a potent model for deciphering general innate immune mechanisms in animals. Investigations on the highly efficient immune reactions in this Dipteran insect have also provided information on other insects that have dramatic repercussions on human life as agricultural pests or as vectors for diseases such as malaria (e.g., the mosquito *Anopheles*).

The completion of the *Drosophila* genomic sequence in 2000 (6) and the subsequent expansion of new postgenomic technologies including proteomics, microarrays, and RNAi (RNA interference) have considerably widened the possibilities of immune system analysis in this model organism. This

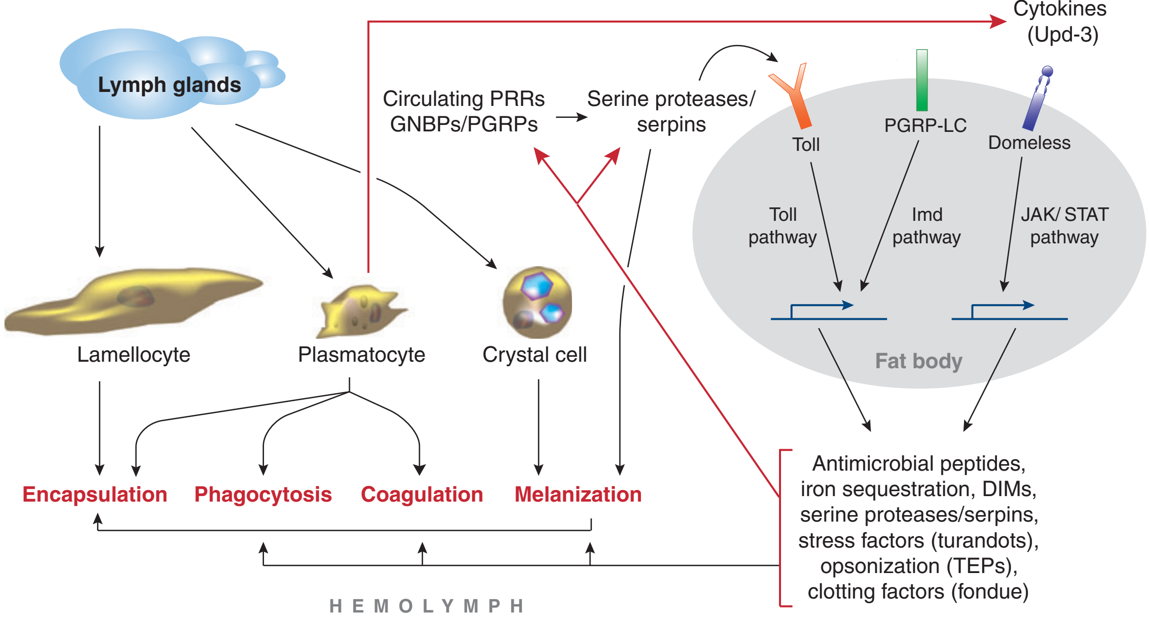

Figure 1

Schematic overview of *Drosophila* host defense. Detection of microbial pathogens elicits a large array of interconnected and synergistic defense modules in immune-responsive tissues.

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article presents an overview of our current knowledge of the *Drosophila* immune response in the context of two fundamental questions: (*a*) What are the molecular mechanisms underlying the defense reactions? (*b*) How does each of these mechanistic modules contribute to defense during an infection, and what strategies have been developed by the pathogens to evade them?

REPERTOIRE OF *DROSOPHILA* DEFENSE MECHANISMS

A hallmark of the *Drosophila* host defense, and that of most other holometabolous insects, is the challenge-induced synthesis and secretion of potent AMPs that accumulate in the hemolymph where they oppose invading microorganisms. Although synthesis of AMPs is probably common to all metazoans, secretion of these molecules into the hemolymph is not a general phenomenon. We refer it to as the "systemic immune response," which is by far the best analyzed among *Drosophila* immune reactions, and analyze it from the synthesis of immune effectors to recognition of infection. Epithelial immunity, i.e., the fight against invading microorganisms at the level of the barrier epithelia, is now understood to significantly contribute to the protection of *Drosophila*. This response is analyzed next both in terms of AMP and reactive oxygen species (ROS) production. A subsequent section deals with the cellular response by the hemocytes, especially their role in phagocytosis and encapsulation of parasites. The final section is devoted to two reactions, coagulation and melanization, which are activated immediately upon injury.

The Systemic Immune Response

Injection of bacteria into the body cavity induces the appearance of antimicrobial activity in the hemolymph of *Drosophila*. This activity persists for several days and can confer protection against a second challenge by pathogenic bacteria (7). This reaction mainly consists of AMP production by the fat body, which is a major immune-responsive tissue that originates from the mesoderm during embryogenesis and acquires its immune competence at the onset of the first larval stage. Due to its large size and its location inside the open circulatory system of the insect body cavity, the fat body represents a powerful organ for the synthesis and secretion of peptides into the hemolymph, where they readily reach their effective concentrations.

ROS: reactive oxygen species

Immune effectors. Among the various molecules produced by the fat body in response to infection, AMPs are the best characterized. Some 20 immune-inducible AMPs, which can be grouped into seven classes, have been identified (**Figure 2**). They are small (<10 kDa), with the exception of the 25 kDa Attacin, and cationic and exhibit a broad range of activities against bacteria and/or fungi (8). Diptericin, Drosocin, and Attacin are very effective against Gram-negative bacteria (9–11). Defensin is active against Gram-positive germs (12), whereas Drosomycin and Metchnikowin are antifungal agents (13, 14). Cecropin A1 acts against both bacteria and some fungi (15, 16). Defensins and Cecropins have been reported from many insects, whereas Drosomycin and Metchnikowin have so far been identified only in Drosophilidae (8). The insect AMPs are membrane-active, and their precise mode of action at the membrane level is still under investigation. Some AMPs are very stable owing to the presence of intramolecular disulfide bridges and are still detected in the hemolymph several weeks after challenge (17). Experiments with transgenic flies overexpressing a single AMP provided support for a critical role of AMPs in resistance to infection in *Drosophila* (18). However, the particular contributions of each of these AMPs have not been tested, as loss-of-function mutants for AMP genes are not available to date.

Recent large-scale analyses, at the transcriptome and proteome levels, have revealed that in addition to that of AMPs, the

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Peptides	# of genes	Main activity	Concentration	3-D structure
Diptericin	2	Gram-negative bacteria	0.5 µM	nd
Attacin	4	Gram-negative bacteria	nd	nd
Drosocin	1	Gram-negative bacteria	40 µM	
Cecropin	4	Gram-negative bacteria	20 µM	
Defensin	1	Gram-positive bacteria	1 µM	
Drosomycin	7	Fungi	100 µM	
Metchnikowin	1	Fungi	10 µM	nd

Figure 2

Drosophila antimicrobial peptides. Name, number of genes in the genome, antimicrobial activities, estimated concentration in the hemolymph after bacterial injection, and 3-D structure (8) (nd, not determined).

production of many peptides and proteins is upregulated after septic injury (19–25).[1] Some of these are involved in the regulation of the systemic immune response itself (e.g., signaling components). Another group of proteins (opsonins, components of the melanization or clotting system) participates in distinct defense mechanisms, while an additional group includes putative immune effectors. Among these are 17 members of the DIM family

DIM: *Drosophila* immune molecule

[1]A list of all *Drosophila* immune genes can be found on the web at **http://www.cgm.cnrs-gif.fr/immunity/enindex.html**.

(*Drosophila* immune molecule) and 8 Turandot proteins, which are small peptides of unknown functions secreted by the fat body (17, 25–27). One catalase gene, two transferrin genes, and one iron transporter gene are also induced following septic injury, pointing to a role of ROS and iron sequestration in the control of microbial development (19, 28). Iron is essential for most invading microorganisms during the course of an infection, and both animals and plants have elaborated immune strategies that limit iron availability to the microorganisms.

Collectively, the systemic antimicrobial response represents a dramatic change in gene expression that not only results in the production of antimicrobial molecules in the hemolymph, but also participates in other immune mechanisms. Major challenges are to understand the relative contribution of these immune effectors to the total host defense, to test their specificity against certain pathogen classes, and to determine potential synergies between them.

Regulation. The massive expression of novel peptides/polypeptides that occurs following infection is primarily regulated at the transcriptional level. The cloning in the early 1990s of the genes in *D. melanogaster* that encode AMPs followed by promoter mapping experiments with the *Cecropin A1* and *Diptericin* genes revealed the presence of DNA motifs required for immune inducibility. These include a combination of NF-κB binding sites, GATA binding sites recognized by the transcription factor Serpent, and a less well-defined motif called R1 (29–33). Prominent among these motifs are the κB response elements, which confer immune inducibility (34–36). Three NF-κB/Rel-like proteins are encoded in the *Drosophila* genome. Dorsal and Dif, encoded by two clustered genes, contain an N-terminal Rel DNA binding domain and a C-terminal transactivator domain, whereas Relish is similar to mammalian p105, consisting of an N-terminal Rel domain and a C-terminal inhibitory ankyrin repeat domain (37–39a). Gel shift assays have shown that the three proteins (Relish, Dorsal, and Dif) bind to κB sites and can transactivate some of the AMP genes in cell culture (37, 38, 40, 41). Furthermore, genetic studies have demonstrated the key roles of these transactivators in the regulation of AMP genes via two distinct signaling pathways, referred to as Toll and Imd pathways.

The Toll pathway. The Toll pathway is an evolutionarily conserved signaling cascade that plays a key role in the establishment of the dorso-ventral axis of the *Drosophila* embryo, as well as in several other developmental processes (42). Canonic components of this pathway are the extracellular cytokine Spätzle (which shares structural similarities with the nerve growth factor, NGF), the transmembrane receptor Toll, the Tube and MyD88 adaptors, the Pelle kinase, Cactus (the *Drosophila* homolog of IκB), and the Dorsal and Dif transactivators (42, 43) (**Figure 3**). Deletion of any of these components (except for Cactus and Dorsal) causes a similar immune-deficient phenotype characterized by the lack of expression of several immune genes, including the antifungal peptide *Drosomycin* gene, and a marked susceptibility to fungal and Gram-positive bacterial infection (44, 44a). Dif and Dorsal seem to play redundant roles in the control of *Drosomycin* at the larval stage, whereas Dif is sufficient to mediate Toll activation in adults (45–47). Subtle roles for Dorsal have been proposed at the adult stage (48). Many components of the Toll pathway are themselves upregulated in a Toll-dependent manner upon infection (44, 49). The induction of the inhibitor molecule, Cactus, establishes a negative feedback on this cascade (50). Unlike vertebrate Toll-like receptors (TLRs), Toll does not function as a pattern recognition receptor but is activated by the cytokine Spätzle (44, 51, 52). The *Drosophila* genome encodes eight other Toll proteins, none clearly implicated in fly immunity (53, 54). The *Drosophila* Toll pathway shares significant similarities

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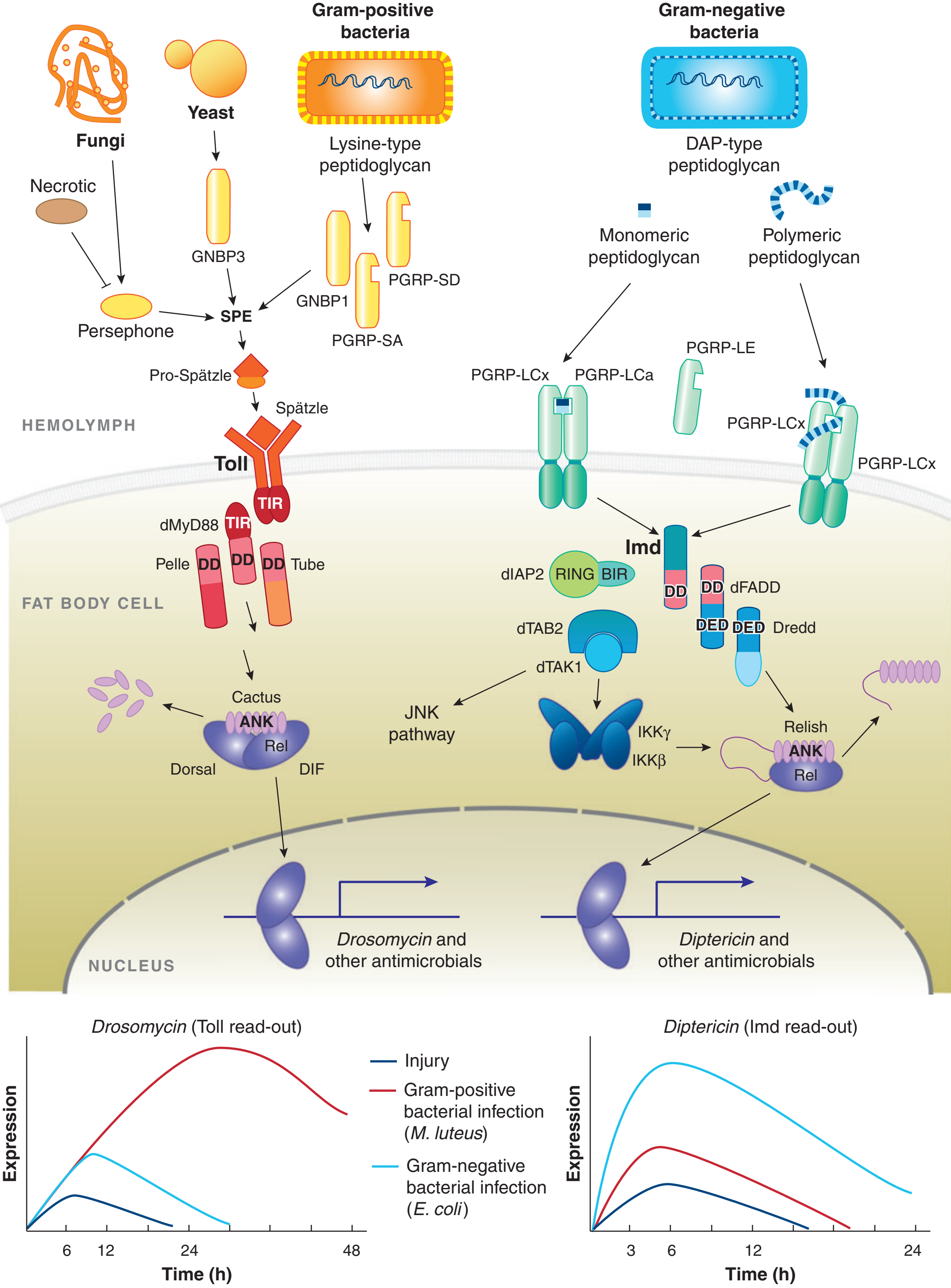

with the signaling cascade activated downstream of Interleukin-1 and the TLRs, pointing to a common ancestry of these immune mechanisms.

The Imd pathway. This pathway was initially defined by the identification of a mutation named *immune deficiency* (*imd*) that impaired the expression of several antibacterial peptide genes but only marginally affected *Drosomycin* induction (55–57). *imd* flies succumb to Gram-negative bacteria but are more resistant to fungi and Gram-positive bacteria than are Toll mutant flies. *imd* encodes a death domain–containing protein similar to that of Receptor Interacting Protein (RIP) of the tumor necrosis factor receptor (TNF-R) pathway, and its overexpression triggers the transcription/induction of antibacterial peptide genes in the absence of an infection (58). Genetic screens and reverse genetic approaches have led to the identification of eight additional canonical components of the Imd pathway: the PGRP-LC receptor (59–61); the Mitogen-Activated Protein 3 kinase (MAP3K) TAK1 (62, 63); TAB2 (64–66); DIAP2, a member of Inhibitor of Apoptosis proteins (64, 65, 67); IKKβ/ird5 and IKKγ/Kenny, which form a *Drosophila* equivalent of the mammalian IKK signalosome (68–70); the dFADD adaptor (71, 72); the Dredd caspase (73); and the transcription factor Relish (74). Mutations affecting these factors generate an immune deficiency similar to that of *imd*. Noninfected *imd*-deficient flies are perfectly viable, and to date no developmental role has been attributed to this pathway. The Imd pathway shares some similarities

TLR: Toll-like receptor

TNF-R: tumor necrosis factor receptor

Figure 3

Model of Toll and Imd pathway activation. (*Top*) Antimicrobial peptide genes are regulated by a balance between two signaling pathways: the Toll pathway that is largely activated by fungi and Gram-positive bacteria, and the Imd pathway that is mainly activated by Gram-negative bacteria. According to the κB sites present in their promoters, antimicrobial peptide genes are more sensitive to either the Toll cascade (e.g., *Drosomycin*) or the Imd cascade (e.g., *Diptericin*) or are coregulated. *Toll pathway*: The Toll receptor is activated upon binding with a cleaved form of Spätzle that is processed by proteolytic cascades activated by secreted recognition molecules (PGRP-SA, PGRP-SD, GNBP1, GNBP3). Three distinct recognition modules that involve the sensing of Gram-positive bacteria (PGRP-SA, PGRP-SD, GNBP1), Glucan (GNBP3), and entomopathogenic fungi (via the direct cleavage of an SP) converge to the activation of SPE that cleaves Spätzle. Mature Spätzle binds as a dimer to Toll, thereby inducing its dimerization at the plasma membrane (51, 52). This causes the recruitment of three intracellular Death domain–containing proteins, MyD88, Tube, and Pelle, the latter also being a kinase (43). By a mechanism still uncharacterized, Cactus is phosphorylated, then degraded by the proteasome (50). As a consequence, the Rel transcription factors Dif and Dorsal are released and move from the cytoplasm to the nucleus (37, 142). Among the less well-understood aspects of this cascade are the respective roles of the multiple Spätzle isoforms, the mechanisms that link Pelle activation to Cactus degradation, and the complex regulation of Cactus isoforms. *Imd pathway*: Upon direct binding with bacterial elicitors (monomeric or polymeric DAP-type PGN), PGRP-LC recruits the adaptor Imd (127, 272). Imd then interacts with dFADD, which itself binds the apical caspase Dredd (273). This caspase has been proposed to associate with Relish, which it might cleave directly once Relish is phosphorylated (274, 275). After Relish cleavage, the Rel domain translocates to the nucleus, whereas the inhibitory domain remains stable in the cytoplasm. Relish is phosphorylated by the IKK signaling complex (68), which is itself thought to be activated by TAK1 and its adaptor TAB2 in an Imd- and possibly dFADD-dependent manner. The Ring domain protein DIAP2 may activate dTAK1. At present the mechanisms that link Imd to the IKK complex and the precise role of TAK1 and DIAP2 are not known (see text). TIR (Toll-IL1 receptor domain), DD (death domain), DED (death-effector domain), ANK (ANKyrin repeats), Rel (Rel homology domain), RING (RING finger domain), BIR (Baculovirus IAP repeat), SPE (Spätzle processing enzyme). (*Bottom*) Differential expression of *Drosomycin* (Toll target) and *Diptericin* (Imd target) genes in response to injection of *E. coli* (Gram-negative bacteria) or *M. luteus* (Gram-positive bacteria) (83). *Diptericin* shows an acute phase profile, whereas *Drosomycin* exhibits a late and sustained expression pattern.

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MAPK: mitogen-activated protein kinase

with the vertebrate TNF-R pathway. Several proteins were recently identified that affect the Imd pathway (64, 75–80a). The molecular organization of the Imd pathway is, however, as yet poorly defined owing to difficulties in performing epistatic analyses and to the absence of data on the subcellular localization of most of its components (**Figure 3**).

Interactions between Toll and Imd pathways. To date, the Toll and Imd pathways are the sole reported intracellular cascades of *Drosophila* that are activated by microbial ligands. Microarray studies using *imd/Toll* double mutant flies have shown that these cascades regulate almost 80% of the genes induced upon septic injury, reflecting their important contribution in survival from infection (49). An open question is whether the Toll and Imd pathways can synergize to increase the levels of induction of some of the immune-response genes, possibly via the formation of Dif/Relish heterodimers. Bioinformatic analysis has recently identified κB binding sites specific for either Toll or Imd pathway activation (81). Although some immune-induced genes are clearly dependent on one pathway only (*Dipterícin* for Imd, *DIM1* for Toll), others can apparently be induced by both cascades. Most notably, *Drosomycin*, which is a convenient read-out for Toll pathway activation, receives a modest input from the Imd pathway in the systemic response and is solely activated by the Imd pathway in the local response (73, 82). The susceptibility of *Toll* and *imd* mutants to different classes of microorganisms is explained by the differential activation of these two pathways by distinct microorganisms as well as by the existence of specific immune target genes. Another important aspect of the antimicrobial response is the temporal activation of these two pathways. Genes regulated by Imd generally show an acute phase profile, whereas Toll target genes exhibit a late and sustained expression pattern (21, 47, 83) (**Figure 3**).

Finally, a number of developmental and physiological factors affect Toll and Imd signaling by influencing the maturation of the fat body. This is reflected by the immune inducibility of *Diptericin* in larvae, which increases with age and is dependent on ecdysone, the key molting hormone of insects (84). All mutations affecting ecdysone metabolism can thus indirectly affect the immune response. This effect complicates immune studies and may explain a number of conflicting reports (85).

The JAK/STAT pathway. The JAK/STAT pathway, originally identified through its role in embryonic segmentation, has three main cellular components: the receptor Domeless, the Janus Kinase (JAK) Hopscotch, and the STAT transcription factor (86). The first evidence for an involvement of the JAK/STAT pathway in insect immunity came from studies performed in the mosquito *Anopheles* indicating that after an immune challenge the STAT protein accumulates in the nucleus (87). Subsequent gene expression profile studies identified a subset of *Drosophila* immune-responsive genes that are regulated by the JAK/STAT pathway, namely the genes encoding the complement-like protein *Tep2* and the *Turandot* stress genes (21, 88, 89). *Turandot* are *Drosophila*-specific genes of unknown function that are induced by various stress conditions, especially by septic injury (26, 27). The transcriptional regulation of these genes is complex, with additional inputs from both the Imd and MAPK (mitogen-activated protein kinase) pathways (90).

JAK/STAT-deficient flies are as resistant to bacterial and fungal infections as are wild-type flies, and they express a normal AMP profile. They are, however, sensitive to infection with the *Drosophila* C virus (91). It has been proposed that the JAK/STAT pathway could respond to tissue damage and that a hemocyte-released cytokine, Unpaired-3 (Upd-3), activates this pathway by binding to the fat body Domeless receptor during infection (89). The precise role of this pathway and its overall contribution to the host defense remain to be established.

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Other pathways. The JNK pathway regulates many developmental processes in *Drosophila* and is required for proper wound healing of the epidermis (92, 93). Microarray analysis of S2 cells, a hemocyte-derived cell line, has shown that TAK1 activates the JNK pathway in response to bacteria via the JNK kinase basket (21). These data are consistent with a model where Imd signaling bifurcates downstream of TAK1, activating both JNK and IKK signaling (**Figure 3**). Some negative feedbacks between the Imd-Relish and Imd-JNK branches have been reported (94, 95). In S2 cells, JNK-dependent immune genes encode many proteins involved in cytoskeleton remodeling, in keeping with a role in hemocyte activation (21). A role for the JNK pathway in AMP gene expression by the fat body has been proposed (96, 97).

The p38 stress pathway has been implicated in the immune response of plants, *Caenorhabditis elegans*, and mammals (98, 99). The *Drosophila* genome encodes two homologs of p38, and cell culture experiments identified several MAPKs acting upstream (100, 101). Although there is no in vivo evidence for a role of this pathway in the antimicrobial response, its implication in immune functions remains possible. Eiger/Wengen are *Drosophila* proteins with similarities to mammalian TNF and TNF-R, respectively (102, 103). Flies carrying a mutation in these genes are fully viable and do not display obvious immune defects. Overexpression of the *eiger* and *wengen* genes, however, leads to apoptosis via activation of the JNK pathway. The physiological function of these proteins has not yet been determined.

Microbial recognition. Injection of Gram-positive or Gram-negative bacteria and fungi induces different patterns of AMP expression, indicating that microbial recognition mechanisms can differentiate between various classes of invaders (83). This property favors adapted effector responses via the selective activation of the Toll and Imd pathways. Microbial detection is a process that ultimately requires direct contact between a host protein called pattern recognition receptor (PRR) and a microbial molecule. Genetic studies have shown that microbial recognition acting upstream of the Toll and Imd pathways is achieved through peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs), two families of PRRs initially identified in larger insects for their capacity to bind microbial ligands and to activate the proPO (prophenoloxidase) cascade (104–106).

PRR: pattern recognition receptor

PGRP: peptidoglycan recognition proteins

GNBP: Gram-negative binding protein

proPO: prophenoloxidase

PGN: peptidoglycan

DAP: *meso*-diaminopimelic acid

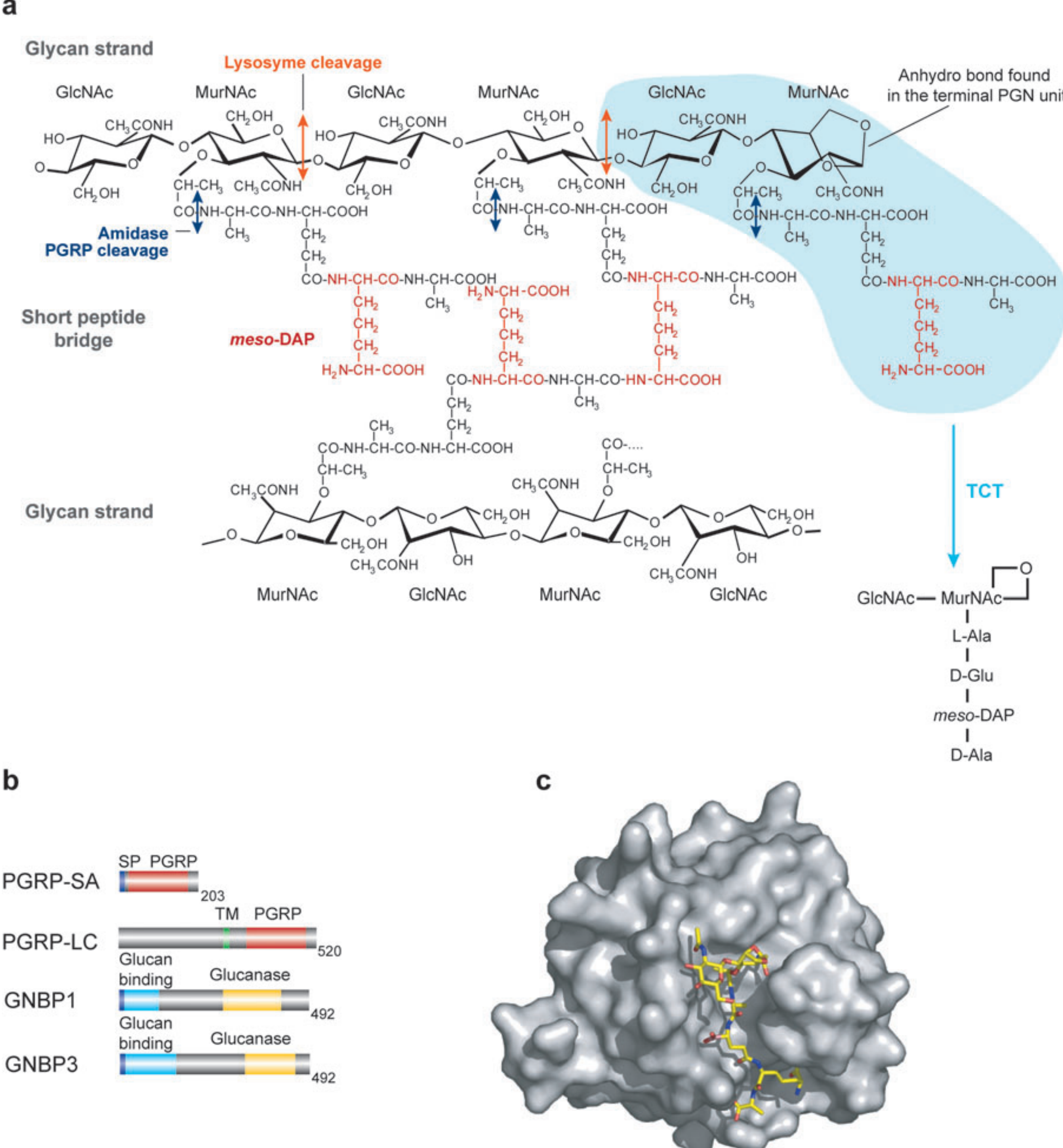
LPS: lipopolysaccharide

Bacterial recognition by PGRPs. In *Drosophila*, recognition of bacteria is achieved through the sensing of specific forms of peptidoglycan (PGN) by PGRPs. PGN is an essential glucopeptidic polymer restricted to the cell wall of both Gram-negative and Gram-positive bacteria (**Figure 4*a***). It consists of long glycan chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues that are cross-linked to each other by short peptide bridges (107). PGN is a highly complex and fast-evolving molecule with marked differences from one bacterium to another. PGN from Gram-negative bacteria differs from most Gram-positive PGN by the replacement of lysine with *meso*-diaminopimelic acid (DAP) at the third position in the peptide chain. There is, however, a subclass of Gram-positive bacteria including *Bacillus* species, which produces DAP-type PGN. Another major difference between Gram-negative and Gram-positive PGN is its localization within the cell wall. Gram-negative PGN consists of a single layer and is hidden in the periplasmic space underneath the outer membrane and lipopolysaccharide (LPS) layer, whereas PGN from Gram-positive bacteria is multilayered and exposed at the bacterial surface.

The Imd pathway is activated by DAP-type PGN, whereas the Toll pathway is activated by Lys-type PGN, as demonstrated by studies using highly purified bacterial compounds (108). In contrast to vertebrates, LPS endotoxin, the major component of the

Gram-negative cell envelope, has no effect on Toll and Imd pathway activity, and previous positive results were explained by the presence of PGN contaminants in commercial LPS preparations (108, 109).

Further studies have demonstrated that both polymeric and monomeric DAP-type PGN can activate the Imd pathway. A specific monomer, GlcNAc-MurNAc(anhydro)-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala monomer, also known as tracheal cytotoxin (TCT), was



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identified as the minimal PGN motif capable of efficient induction of the Imd pathway (109, 110). TCT provides an ideal "signature" of Gram-negative bacteria because this muropeptide is located at the end of the PGN strand and is released from PGN during cell growth and division (**Figure 4*a***). In contrast, the minimum structure needed to activate the Toll pathway is a muropeptide dimer of lysine-type PGN (111).

PGRPs are highly conserved from insects to mammals and share a 160 amino acid domain (the PGRP domain) with similarities to bacteriophage T7 lysozyme, a zinc-dependent *N*-acetylmuramoyl-L-alanine amidase (112–114) (**Figure 4*b***). Sequence analysis of the 13 *Drosophila* PGRPs points to the existence of two subgroups with either recognition or enzymatic properties. Members of the first group (PGRP-SA, SD, LA, LC, LD, LE, and LF) lack zinc-binding residues required for amidase activity but still retain the ability to bind and recognize PGN and function as PRRs. In contrast, proteins from the other subgroup of PGRPs, referred to as catalytic PGRPs, have demonstrated (PGRP-SC1, LB, SB1) or predicted (SC2, SB2) zinc-dependent amidase activity that removes peptides from the glycan chains, thereby reducing or eliminating the biological activity of PGN (115–116a). Some of these PGRPs modulate the immune response by scavenging PGN (115–117). The tertiary structures of four PGRPs have now been solved (118–123). A prominent feature is the presence of an extended surface groove in the PGRP domain, which includes a zinc-finger cage in the catalytic PGRP-LB. The structures of PGRP-LE and PGRP-LCa/x in complex with TCT show an interaction between the peptide stem of PGN and the PGRP groove (**Figure 4*c***). In contrast to other PGRPs, the PGRP domain of PGRP-LCa does not possess a typical PGN-docking groove, in agreement with its role as a coreceptor sensing monomeric DAP-type PGN (121).

Recognition of Gram-negative bacteria by PGRP-LE/LC. PGRP-LC-deficient flies display a strongly reduced expression of antibacterial peptide genes in response to Gram-negative bacteria. This effect is similar to, albeit weaker than, that observed for *imd*-deficient mutants. Epistatic and phenotypic analyses indicated that PGRP-LC is the major receptor of the Imd pathway (59). Alternative splicing of PGRP-LC can produce three proteins (LCa, LCx, LCy) that share the same intracellular (signaling) domain but have distinct extracellular (sensing) domains (109, 112). Studies in cell culture using RNAi specific for each isoform have shown that PGRP-LCx is required for recognition of polymeric PGN, whereas both PGRP-LCa and PGRP-LCx are mandatory for detection of monomeric PGN (109). The current belief is that signaling is achieved by association of at least two PGRP-LC molecules in close

Figure 4

Recognition upstream of the Toll and Imd pathways. (*a*) Structure of *E. coli* PGN. PGN is a complex heteropolymer consisting of long glycan chains of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues, connected by short peptide bridges (107). All the terminal MurNAc residues ending the *E. coli* PGN glycan chains have a unique internal 1,6-anhydro bond. The TCT monomer (indicated in *blue*) occurs naturally in Gram-negative bacteria at the extremity of all glycan strands and is consequently present in approximately 5% of the GlcNAc-MurNAc units. Lysosymes catalyze the cleavage of the β-1,4-glycosidic bond between the MurNAc and GlcNAc residues (*orange arrow*) while amidase PGRPs remove the peptidic bridge from the sugar backbone (*blue arrow*). (*b*) Schematic structure of some PGRPs and GNBPs. SP, signal peptide; TM, transmembrane domain; PGRP, PGRP domain. GNPBs have an N-terminal domain that has been shown to bind β(1,3)-glucan and a C-terminal domain with homology to glucanase. (*c*) Three-dimensional structure of PGRP-LE with TCT (120). A prominent feature is the presence of an extended surface groove in the PGRP domain that interacts with the peptide stem of TCT (*in color*).

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proximity through binding of polymeric PGN. Such an interaction cannot occur with monomeric PGN, and in this case PGRP-LCa is expected to act as an adaptor (121, 124). This model is supported by the crystallization of TCT in complex with both PGRP-LCa and LCx (122).

PGRP-LE encodes a PGRP with affinity to DAP-type PGN and is expressed both extra- and intracellularly (125, 126). A fragment of PGRP-LE corresponding to the PGRP domain alone functions extracellularly to enhance PGRP-LC-mediated PGN recognition on the cell surface, a role evocative of that of mammalian CD14 in binding of LPS to TLR4 (127). A full-length form of PGRP-LE is also present in the cytoplasm and acts as an intracellular receptor for monomeric PGN, thus bypassing the requirement for PGRP-LC (127). Monomeric PGN probably gains access to the cytoplasm by virtue of its small size. Thus, both PGRP-LC and PGRP-LE could participate in the sensing of Gram-negative bacteria upstream of the Imd pathway (**Figure 3**).

Downregulation of the Imd pathway by amidase PGRPs. Injection of Gram-negative bacteria induces a transient expression of antibacterial peptide genes (**Figure 3**), suggesting the existence of a mechanism to downregulate the immune response. Recent studies in *Drosophila* revealed a key role for amidase PGRPs in controlling the level of Imd pathway activity by degrading PGN (116, 117). PGRP-LB and PGRP-SC are efficient amidases that remove peptides from the glycan chains and thereby convert Gram-negative PGN into nonimmunostimulatory fragments. PGRP-LB is active only on DAP-type PGN, whereas PGRP-SC digests both DAP- and Lys-type PGN (115, 116). RNAi extinction of *PGRP-SC1/2* or *PGRP-LB* leads to higher *Diptericin* expression after systemic infection compared to that of wild-type flies. *PGRP-LB* encodes a secreted protein and is regulated by the Imd pathway. The presence of PGRP-LB in the hemolymph establishes a negative feedback loop that ensures an appropriate degree of immune activation in response to bacterial infection (116). This mechanism economizes host resources by anticipating the termination of the immune response. Amidase PGRPs may also prevent potentially severe consequences to host tissues through prolonged immune activity. In agreement with this idea, *PGRP-SC1/2* RNAi larvae infected with bacteria showed increased lethality associated with developmental defects (117). The *Drosophila* genome encodes four other catalytic PGRPs. A combination of various amidase PGRPs likely ensures an adequate degree of immune reactivity in each fly tissue.

Recognition of Gram-positive bacteria. A mutation in *PGRP-SA* blocks the activation of the Toll pathway by Gram-positive bacteria but affects neither Toll activation by fungi nor Imd pathway activation by Gram-negative bacteria (128). PGRP-SA encodes a secreted PGRP, which is present in the hemolymph and acts upstream of Toll in the recognition of Lys-type PGN of Gram-positive bacteria (**Figure 4*b***). Two additional proteins, GNBP1 (Gram-negative Binding Protein, in fact a historical misnomer) and PGRP-SD, have been implicated in the sensing of Gram-positive bacteria. The immune phenotype of loss-of-function mutations in *GNBP1* is indistinguishable from that of *PGRP-SA* mutations, and experimental data indicate that GNBP1 and PGRP-SA form a complex in the hemolymph (129, 130). A proposed function of GNBP1 would be to hydrolyze Gram-positive PGN into small fragments susceptible to detection by PGRP-SA (111, 130a). Some Gram-positive bacterial species such as *Staphylococcus aureus* can activate the Toll pathway in a PGRP-SA- and GNBP1-independent manner, indicating that alternative receptors exist. Consistent with this hypothesis, PGRP-SD, yet another secreted PGRP, was found to mediate Toll activation by a subset of Gram-positive bacteria, in partial redundancy with the PGRP-SA/GNBP1 complex (131).

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Fungal recognition. Recent studies point to a role of GNBP proteins in the detection of fungal infection (81). Members of this family display a significant overall homology to bacterial glucanases (131a) (**Figure 4*b***). They contain an N-terminal domain that binds to β(1,3)-glucan and a C-terminal domain that is homologous to the catalytic domain of β-glucanase. However, the absence of conserved key residues in the catalytic site suggests that this domain is not functional. The *Drosophila* genome contains three consensus members of the GNBP family and three related members containing only an N-terminal domain similar to that of GNBP3. The GNBP3 protein sequence is highly similar to that of lepidopteran β Glucan Recognition Proteins known to bind fungal β(1,3)-glucans (131b). A role of GNBP3 in fungal detection is supported by the observation that *GNBP3* mutants are sensitive to fungal infection and fail to activate the Toll pathway (131c). According to this observation, GNBP3 is a circulating PRR acting upstream of the Toll pathway in fungal detection.

Activation of Toll by serine proteases. The Toll receptor is activated upon binding of a cleaved form of Spätzle (51, 52). The immune-induced cleavage of Spätzle is thought to be triggered by secreted PRRs that activate a proteolytic cascade, a process conceptually similar to vertebrate blood coagulation or complement activation cascades (132). These proteolytic cascades have a functional core consisting of several serine proteases (SPs) that undergo zymogen activation, followed by cleavage of a substrate by the terminal downstream protease. SP cascades allow the shaping and amplification of extracellular signals and are involved in complex regulatory mechanisms, in which inappropriate activation is prevented by the action of SP inhibitors (serpins) (133). The *Drosophila* genome encodes more than 200 SPs and serine protease homologs (SPHs), most of which have as yet uncharacterized functions (134). SPHs represent one quarter of all *Drosophila* SP-related proteins and are thought to possess regulatory functions (135, 136). Many SPs that function in insect immunity contain a Clip-domain N-terminal to the catalytic domain (137). This domain is only found in insect SPs and is believed to play a regulatory role in the sequential activation of SPs.

SP: serine protease

SPH: serine protease homolog

The activation of Spätzle during the immune response is achieved by a set of SPs distinct from those mediating Toll activation during embryonic development (44). Recent studies demonstrate the existence of a common core of SPs and SPHs acting upstream of Spätzle in the immune response that integrate signals sent by various secreted recognition molecules via more specialized SPs (138). SPE, an immune-regulated Clip-domain SP, has been identified as the Spätzle processing enzyme (138, 139). SPE presents 89% amino acid identity with Easter, the enzyme that processes Spätzle in the embryo. Toll activation by entomopathogenic fungi is independent of PGRP-SA, but is mediated by an extracellular cascade involving the Clip-SP Persephone (140) and the inhibitory serpin Necrotic (141). Overexpression of *persephone* or loss of function of *necrotic* is sufficient to trigger Spätzle-dependent activation of Toll in the absence of an immune challenge.

Recognition: concluding remarks. The past few years have seen the identification of the *Drosophila* PRRs regulating the Toll and Imd pathways and of the microbial ligands that they recognize. From an evolutionary point of view, it is interesting to note that insects use GNBPs and PGRPs as PRRs because these are proteins derived from enzymes known to degrade microbial cell wall products. Thus, the roles of these molecules may have evolved from microbicidal to recognition.

The organization of the Toll and the Imd pathways differs, in that the former is activated by secreted PRRs whereas the latter is activated through membrane-bound PRRs. This difference of architecture could reflect the mode of recognition: (*a*) direct for the Toll pathway for which PRRs can bind to

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microbial ligands exposed at the surface of Gram-positive bacteria and fungi, or (*b*) indirect for the Imd pathway, which is activated by PGN released from the periplasm of Gram-negative bacteria.

Finally, it cannot be formally excluded that these pathways are activated through the recognition of host molecules (e.g., molecules released at the wound site). The existence of endogenous ligands is also supported by the observation that larvae with melanotic tumors display a significant AMP gene expression level in the fat body in the absence of an infection (142–144). One report suggests that endogenous DNA can activate the Imd pathway based on the observation that a fly line deficient in DNase II exhibits a higher *Diptericin* expression level in the absence of an infection compared to that of wild-type flies (145).

Epithelial Immunity in *Drosophila*

Because the barrier epithelia are in constant contact with large numbers of microorganisms, these surfaces must be armed with efficient systems for microbial recognition and control. This is especially true for insects such as *Drosophila*, which live on decaying matter and feed on fermenting medium. In *Drosophila*, both the gut and trachea, two main routes of infection, are lined with a chitinous matrix. Furthermore, the gut lumen is an environment hostile to microbial colonization due to its physical and physiological properties and the secretion of lysozymes (146, 147). In addition, local production of AMPs and ROS provides two complementary inducible defense mechanisms in the gut (**Figure 5**).

Local AMP expression. Expression of AMPs has occasionally been reported in epithelia of other insect species. In the silkworm larva *Bombyx mori*, *Cecropin* transcription is induced in the epithelial cells underlying the cuticle, when it is lightly abraded in the presence of live bacteria (148). In *Drosophila*, the use of GFP reporter transgenes has revealed that AMP genes are expressed in several surface epithelia that are in contact with the external environment (82, 149). These include the epidermis, the reproductive system, the respiratory tract, and the digestive tract. This AMP synthesis is referred to as the local immune response as opposed to the systemic response. One can distinguish between constitutive and inducible AMP expression in epithelia. In the first case, the AMP gene is expressed constitutively in a defined tissue, and its transcription is not upregulated during microbial infection. This is the case for *Drosomycin* in salivary glands and in the female spermatheca, and for *Cecropin* in the male ejaculatory duct (82). This constitutive expression is not regulated by NF-κB pathways but by various tissue-specific transcription factors such as the homeobox-containing protein Caudal (150, 151). In virgin females, mating stimulates the expression of some AMP genes (152).

The second form is the inducible local AMP gene expression. This response is triggered upon natural infection by Gram-negative bacteria and is mediated by the Imd pathway (82, 153). For example, *Drosomycin* and *Diptericin* are induced in both trachea and gut via the Imd pathway in response to local infection by bacteria such as *Erwinia carotovora* (154). Like the systemic response, the local immune response is mediated through the recognition of Gram-negative PGN by PGRP-LC (116). It remains to be clarified why commensal bacteria or ingested PGN does not generate a state of permanent immune activation in tissues such as the gut. A central role in bacterial tolerance of the gut has been attributed to amidase PGRPs, as they scavenge PGN released by commensals (116, 117) (**Figure 5**). Because Gram-negative PGN is hidden in the periplasmic space underneath the outer LPS membrane and bacteria residing in the gut have a low division rate, commensals may well release only low amounts of PGN that can readily be hydrolyzed by amidase PGRPs. This implies the important concept of a threshold response for local immune activation in order to

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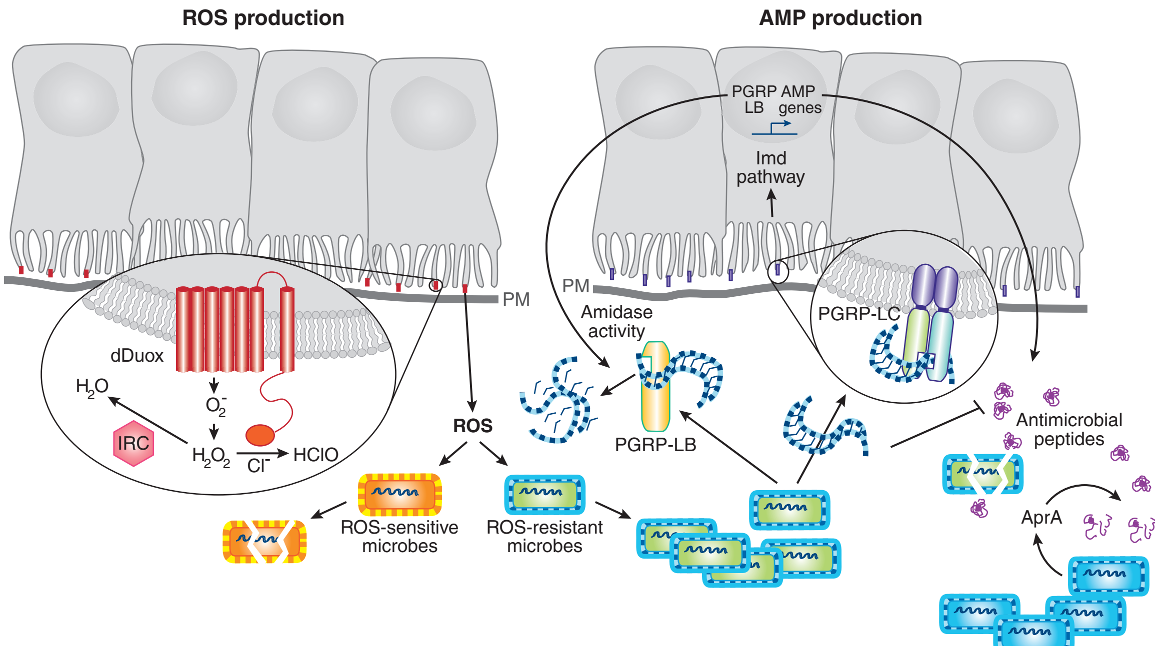

Figure 5

Gut immune response. Local production of ROS (*left*) and AMPs (*right*) provides two inducible defense mechanisms in the gut. ROS are produced by the Duox protein and are detoxified by the IRC catalase (156–158). Enzymatic assays demonstrated that the PHD domain of *Drosophila* DUOX can transform H_2O_2 into the highly microbicidal HOCl. AMPs (e.g., Diptericin or Attacin) are produced by epithelial cells under the control of the Imd pathway upon recognition of PGN released by Gram-negative bacteria (116, 117). Amidase PGRPs degrade PGN in nonimmunostimulatory fragments, thereby reducing the gut immune reactivity. Bacteria such as *Pseudomonas entomophila* secrete an abundant protease (AprA) that degrades AMPs (238). PM, peritrophic matrix.

differentiate between commensal microorganisms and invading pathogens.

To date, no implication of the Toll pathway in the local immune response has been demonstrated, and there is no evidence that AMPs are induced in epithelia in response to Lys-type Gram-positive bacteria or fungi.

ROS production. In mammals an immediate epithelial response to pathogen assault is the generation of ROS. In *Drosophila*, natural infections with bacteria also induce rapid ROS synthesis in the gut, and the dynamic cycle of ROS generation and elimination appears to be vital.

The Duox proteins form a conserved family of molecules that contain, in addition to the NADPH domain, an N-terminal extracellular peroxidase domain (PHD) that can produce ROS in a regulated manner (155). Inactivation of the *Duox* gene by RNAi blocks ROS production in the gut of infected flies, suggesting that this enzyme is the sole source of epithelial ROS (156). *Duox* RNAi flies rapidly succumb to oral infection by the Gram-negative bacteria *E. carotovora*, and this lethality is associated with an inability to control bacterial growth. Excessive ROS production, which is deleterious to the host, is prevented in *Drosophila* by immune responsive catalase (IRC). Silencing of *IRC* by RNAi results in higher ROS production and fly lethality, indicating that IRC provides an antioxidant defense system in *Drosophila* (157). The *IRC* and *Duox* phenotypes demonstrate that a fine redox balance

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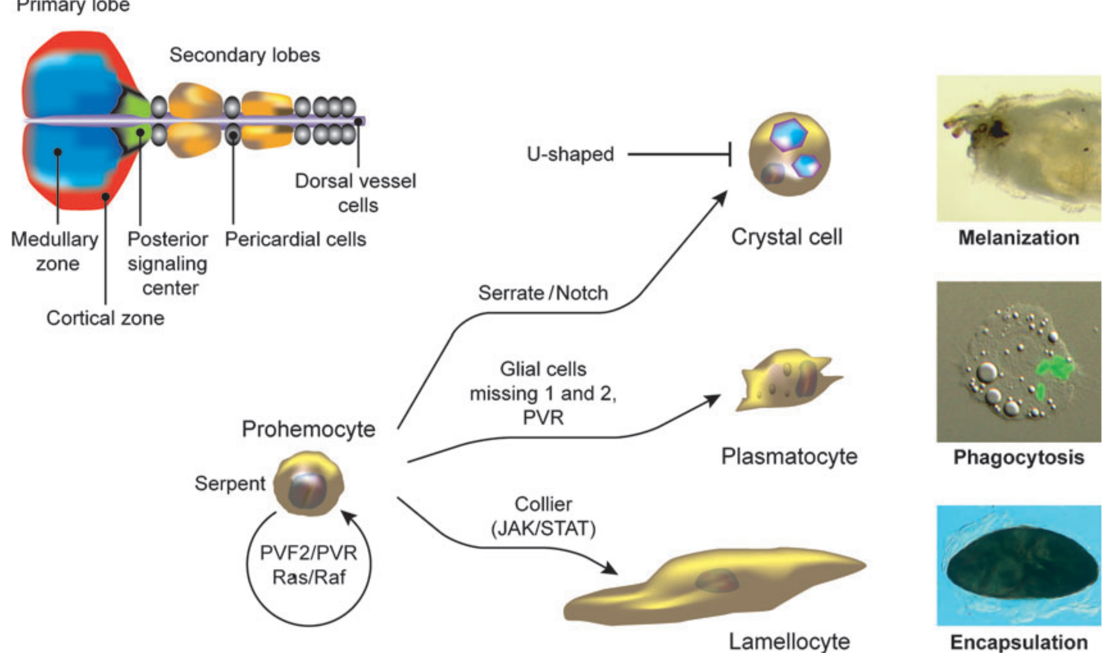

Figure 6

Drosophila hematopoiesis and hemocyte functions. The lymph gland contains a large number of hemocyte progenitors that can differentiate into three hemocyte types with distinct functions (indicated on the *right*). Many factors that regulate key steps of hematopoietic lineage commitment have been identified: serpent (GATA factor), U-shaped (Friend-of-GATA), Lozenge (Runx1 homolog), Glial Cells Missing 1 and 2, PVR (homologous to RTK receptors), and Collier (EBF ortholog) (164, 165, 193). Given their similarities to mammalian hematopoietic factors, these studies point to a conservation of molecular bases for blood-lineage determination in mammalian and *Drosophila* hematopoiesis. The JAK-STAT, Ras/Raf/MAPK, and Toll signaling pathways have also been implicated in hemocyte differentiation and proliferation, but their exact function is not yet known (276, 277).

is critical for control of microorganisms in the gut lumen. This ROS-dependent gut immunity is not affected by the Imd pathway and provides an additional barrier against ingested microorganisms (158) (**Figure 5**).

Cellular Response

The body cavity of *Drosophila*, like that of all arthropods, is filled with a circulating hemolymph that contains both free-floating and sessile blood cells (hemocytes). *Drosophila* larvae contain several thousand hemocytes, which can be divided into the following three cell types on the basis of their structural and functional features: plasmatocytes, crystal cells, and lamellocytes (**Figure 6**) (159, 160). Plasmatocytes represent 90%–95% of all mature larval hemocytes and function in the phagocytic removal of dead cells and microbial pathogens. Lamellocytes are relatively large, flat, and adherent cells that primarily function in encapsulation and neutralization of objects too large to be phagocytosed. Lamellocytes are not found in embryos and adults, and they are rarely observed in healthy larvae, although large numbers of these cells can be induced to differentiate from hemocyte precursors upon infection of larvae with parasitoid wasp eggs. Crystal cells constitute 5% of the larval hemocytes and are nonphagocytic cells involved in the melanization process. Mature crystal cells express proPOs, which are oxidoreductases related to hemocyanins

and which mediate melanization. They are fragile, readily disrupt, and release their contents into the hemolymph upon activation. They thus function as storage cells for the large amounts of proPO present in their cytoplasm in crystallized form (161).

Hematopoiesis. In *Drosophila*, mature plasmatocytes arise from two spatially and temporally distinct phases of hematopoietic development, one early in the embryonic head mesoderm and another during larval development in a specialized organ called the lymph gland (162–166). The embryonic phase of hematopoiesis gives rise to the mature circulating hemocytes of larval stages whereas lymph gland plasmatocytes, under normal, nonimmune conditions, do not enter circulation until metamorphosis. At the onset of metamorphosis, the lymph gland releases a large number of actively phagocytosing plasmatocytes, called pupal macrophages, which play a critical role in tissue remodeling as they phagocytose doomed larval cells. Once in circulation, these lymph gland–derived hemocytes, along with a subset of hemocytes derived from embryonic cells, persist into the adult stage. Many factors that regulate hemocyte differentiation and development have been investigated both at embryonic and larval stages (164, 165) (**Figure 6**). Recent studies have revealed that the primary lymph gland lobes are compartmentalized into three distinct zones: a posterior signaling center that acts as an organizer, a medullary zone containing hemocyte precursors, and a cortical zone containing differentiating hemocytes (**Figure 6**) (167, 168). The posterior secondary lobes serve as a reservoir for prohemocytes in a semiquiescent phase, but have a less well-defined organization than the primary lobes. Hemocyte proliferation is observed mostly in the cortical zone of the lymph gland and is barely detected among circulating cells. Both proliferation and differentiation can be modulated by developmental and immune stimuli. In the adults, lymph glands are absent and no hemocyte proliferation is observed, leaving a uniform population of several thousand mature plasmatocytes.

Blood cell immune functions. Phagocytosis and encapsulation are two major mechanisms of the cellular response.

dsRNA: double-stranded RNA

Phagocytosis. In *Drosophila*, plasmatocytes are responsible for the disposal of both microorganisms and apoptotic cells. They can internalize a large variety of particles such as bacteria, yeast, Sephadex beads, double-stranded RNA (dsRNA), or ink particles within minutes. The first step of phagocytosis is the attachment of the phagocyte to the targeted particle, followed by cytoskeleton modification, internalization, and destruction of the engulfed target within phagosomes.

To date, phagocytosis has been shown to involve several types of receptor proteins. These include members of the scavenger receptor family (dSR-CI), the EGF-domain protein Eater, and the IgSF-domain protein Dscam (169–172). Proteins related to CD36 appear to favor phagocytosis, and two reports have proposed that PGRP family members could also play a role in this process (61, 173, 174). The immune role of phagocytosis receptors is particularly well documented in the case of Eater, which is expressed exclusively on plasmatocytes (and prohemocytes) where it binds to and helps to internalize a broad range of bacteria (171). Importantly, *Eater*-deficient flies show a severe reduction of phagocytosis of Gram-negative and Gram-positive bacteria. An LPS recognition protein (LRP) with six EGF repeats was recently identified in the beetle *Holotrichia diomphalia* (175). LRP is a protein that is secreted into the hemolymph and aggregates Gram-negative bacteria through its association with LPS. This points to the role of EGF-like repeat-containing proteins to phagocytose and aggregate bacteria in insects.

The existence of entry receptors specific for certain pathogens is supported by the observation that knock-down of *pestes*, a member of the CD36 family of scavenger

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TEP: thioester-containing protein

receptors, blocks entry of *M. fortuitum* and *L. monocytogenes* in S2 cells, whereas it does not affect uptake of *E. coli* or *S. aureus* (176).

Another receptor that binds microorganisms and participates in phagocytosis is Dscam. It encodes a member of the Ig superfamily with essential function in neuron interconnection (172). The *Dscam* gene comprises a cluster of variable exons flanked by constant exons, which can theoretically generate by alternative splicing as many as 19,000 isoforms. Secreted isoforms of *Dscam* were detected in the hemolymph, and hemocyte-specific *Dscam* silencing reduces the phagocytic uptake of bacteria. The molecular diversity of *Dscam* transcripts is highly conserved across major insect orders, pointing to a conserved role of this gene.

Systematic RNAi screens in S2 cells identified many *Drosophila* genes required for the internalization of various microorganisms (61, 176–179). As expected, a large number of these genes affect actin cytoskeleton organization (cdc42, Arp2/3complex, actin capping proteins, cofilin) and vesicle trafficking as well as other essential cell functions. Among the rare in vivo studies, a genetic analysis has clearly demonstrated a role for the WASp homolog D-Scar in phagocytosis, while Chickadee, the *Drosophila* homolog of the G-actin sequestering protein Profilin, negatively regulates this process (180). The observation that phagocytosis was only reduced by 40% in *D-Scar* flies underlines the complexity of the phagocytosis process, and the possibility that multiple internalization pathways coexist in *Drosophila* hemocytes.

In mammals, the engulfed target is destroyed within phagosomes by lysosomal enzymes, ROS, and nitric oxide as well as antimicrobial factors such as defensins. The mechanisms that kill the microorganisms inside the vacuoles have been poorly investigated in *Drosophila*. DNase II enzymes are highly conserved proteins that are required for the degradation of DNA within phagolysosomes. Flies depleted in DNase II show an increased susceptibility to infection with both Gram-positive and Gram-negative bacteria, although this phenotype could not be definitely linked to a phagocytosis defect (181).

Opsonization. The *Drosophila* genome harbors six genes coding for proteins structurally related to the complement alpha2-macroglobulin family (88). Five of these genes contain a canonical thioester motif and are referred to as thioester-containing proteins (TEP1 to TEP5); the sixth member, TEP6 or Mcr (Macroglobulin-complement related), lacks the cystein residue that forms the characteristic thioester of TEPs. The TEP family members possess a signal peptide indicating that they are secreted, and three of them, TEP1, TEP2, and TEP4, are upregulated upon infection (88). It has been proposed that TEPs function during the immune response as opsonins to promote phagocytosis and/or protease inhibitors. An opsonin function for a TEP member has been documented in *Anopheles gambiae* where TEP1 is also involved in parasite killing of *Plasmodium* (182). Recently, the contribution of *Drosophila* TEPs to phagocytosis has been investigated in S2 cells using RNAi (178). TEP2 and TEP3 are required for efficient phagocytosis of the Gram-negative bacteria *E. coli* and the Gram-positive bacteria *Staphylococcus aureus*, respectively, and Mcr for successful binding to the surface of *C. albicans* and for efficient internalization of this fungus. Other opsonization factors could be secreted isoforms of Dscam or so far uncharacterized proteins related to the LRP of *H. diomphalia*.

Encapsulation. Encapsulation is a dramatic defense reaction against invading parasites that is mediated by lamellocytes in *Drosophila* larvae. The encapsulation reaction has essentially been analyzed using wasps that lay their eggs into the hemocoel of larvae, but it can also be induced by the injection of an oil droplet (**Figure 6**, and see **Figure 8*b*,*c***) (165, 183). The wasp egg is detected by plasmatocytes, which exert a permanent immune

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surveillance in circulation (184). They attach to the egg chorion and induce within a few hours, through unknown signaling molecules, a strong cellular reaction in the lymph gland, with an increase in proliferation and the massive differentiation of lamellocytes from prohemocytes of the medullary zone and secondary lobes (168). Lamellocytes are released from the lymph gland and then form a multilayered capsule around the invader, a process that is ultimately accompanied by blackening of the capsule due to melanization. Within the capsule, the parasite is eventually killed, possibly by the local production of cytotoxic products such as ROS and intermediates of the melanization cascade (185), but the exact cause of death is not known. The molecular mechanisms underlying the whole process of encapsulation are virtually unknown. Use of thermosensitive alleles of the *myospheroid* gene, which encodes an integrin subunit, reduced the efficiency of capsule formation without affecting lamellocyte differentiation. A role for integrins in this process is not unexpected as lamellocytes aggregate and stick together through septate junctions in order to build a capsule (186). This is further supported by studies in Lepidoptera (187). Two members of the Rho GTPase family, Rac1 and Rac2, which regulate many aspects of cytoskeleton remodeling, have also been shown to participate in this process. In *Rac2* mutants, plasmatocytes adhere to the parasitoid egg but fail to spread, and septate junctions do not assemble, possibly due to defective localization of the Protein 4.1 homolog Coracle (188). Rac1 and the Jun Kinase Basket regulate the formation of actin- and focal adhesion kinase (FAK)-rich placodes in hemocytes and are both required for the proper encapsulation of parasitoid wasp eggs (189). Hemese is a transmembrane glycophorin-like protein with an expression restricted to the cell surface of hemocytes and to the hematopoietic organs (190). Depletion of *Hemese* by RNAi has no obvious effect under normal conditions, but the cellular response to parasitic wasps is much enhanced, suggesting a modulatory role in the activation or recruitment of hemocytes. Microarray analysis identified many genes whose expression is upregulated after wasp infection. These genes are promising candidates for the analysis of the encapsulation process (191).

Encapsulation is a fascinating immune reaction that requires communication between distant organs and involves different hemocyte lineages. The mechanism by which the wasp eggs are recognized by the *Drosophila* immune system is not known. Since parasitoids and *Drosophila* are phylogenetically related, wasp eggs may not be easily detected through PRRs in contrast to fungi or bacteria. Early experiments showed that *Drosophila* larvae encapsulate transplanted tissues from the same species when they are mechanically damaged. Tissue fragments with intact basement membrane remain free in circulation (192). This suggests that the destruction of the basement membrane is sufficient to induce an encapsulation reaction. Hemocytes could recognize intruders due to the absence of a factor found on their own basement membrane. A further question is the nature of the signaling molecules that trigger lamellocyte differentiation within the lymph gland. An attractive hypothesis holds that upon sensing the wasp egg, plasmatocytes send a cytokine to the posterior signaling center in the lymph gland to induce lamellocyte specification in neighboring cells (193).

Melanotic pseudotumors. The melanotic pseudotumor—or melanotic tumor—phenotype is characterized by the presence of black bodies either free-floating within the body cavity, or attached to internal organs (194, 194a). These noninvasive pseudotumors are rare in wild-type flies but can be frequent in some genetic backgrounds. Melanotic capsules share many features with capsules that form around a parasite, as they contain layers of melanized lamellocytes. Watson has proposed a distinction between two types of melanotic tumor mutants (195). Class 1 includes mutants in which melanotic tumors

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result from an "autoimmune response" or the response of an apparently normal immune system to an abnormal target tissue. It was proposed that disruption of the basement membrane that lines tissues could lead to melanotic tumors by inducing hemocyte adhesion and capsule formation (192). Class 2 mutants display overactivation of hemocytes resulting in the formation of capsules. Many mutations known to activate the fly immune system (such as Toll and JAK gain-of-function mutations) belong to this class (142, 196).

Other hemocyte immune functions. Like the fat body, hemocytes store various molecules that can be released upon infection. Plasmatocytes express immune molecules such as the blood clotting factor Hemolectin or the Toll ligand Spätzle, whereas crystal cells contain the enzymes required for the melanization cascade (161, 186, 197). Circulating plasmatocytes express many components of the extracellular matrix (Collagen IV, Peroxidasin, etc.) and may contribute to the formation of basal membranes (198).

Septic injury also triggers the expression of antibacterial peptide genes via the Imd pathway in a subset of circulating plasmatocytes (199). Their contribution to hemolymph antimicrobial activity is probably minimal. Both S2 and mbn-2 cell lines that derive from hemocytes express AMP genes in the presence of Gram-negative PGN and *Drosomycin* in response to Spätzle (43, 110, 200). In these cells, the Imd pathway also activates the JNK pathway that may participate in hemocyte activation through cytoskeleton remodeling (21, 96).

Hemocytes are believed to play signaling functions between distant immune-responsive tissues, in particular via the production of cytokines (e.g., Upd-3) (89).

COAGULATION AND MELANIZATION

Physical breakage of the arthropod cuticle immediately induces hemolymph clotting and melanization. Although these reactions are well characterized in other arthropods (201, 202), progress in our understanding of these reactions in *Drosophila* has been made only recently.

Coagulation

Clotting is critical in limiting hemolymph loss and initiating wound healing in insects as in vertebrates. It is also an important immune defense, quickly forming a secondary barrier to infection, immobilizing bacteria and thereby promoting their killing. In *Drosophila* larvae, a clot composed of fibers trapping hemocytes is rapidly generated at the site of injury (**Figure 7*a*,*c***). This reaction is independent of melanization because it still occurs in proPO-deficient mutants (see below) (203). It is, however, assumed that cross-linking enzymes, including proPO itself and transglutaminase, may be involved in hardening of clots (204, 205). Subsequent steps in wound closure include melanization and epithelial movements (**Figure 7*c***) (92, 93). One plasmatocyte-specific gene, *hemolectin*, has been demonstrated to be required for efficient clot formation in *Drosophila* (197, 203). Hemolectin is a large protein with several domains that are also present in other clotting factors (**Figure 7*b***). It is a major component of the fibers (**Figure 7*b***). A pull-down assay and proteomic studies have identified additional proteins present in the clot (203, 204). One of these, Fondue, is an abundant hemolymph protein regulated by the Toll pathway, which exhibits multiple repeat blocks. Depletion of *fondue* by RNAi induced clotting defects (144). Fondue is not involved in the formation of primary clot fibers, but rather in the subsequent cross-linking of these fibers. Wounding of *fondue-RNAi* or *hemolectin* larvae or flies does not lead to increased mortality compared to challenged controls. Thus it appears that impairment of clotting in vivo leads to more subtle phenotypes, such as the formation of larger scabs (144, 203).

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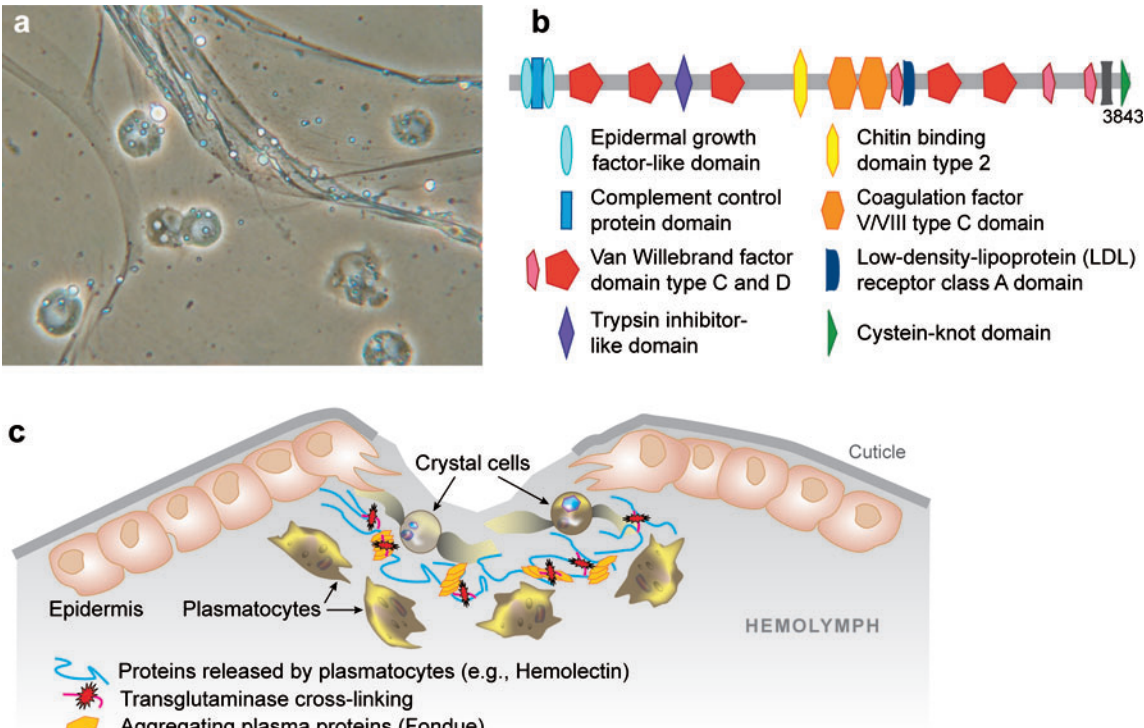

Figure 7

Clotting reaction in *Drosophila*. (*a*) A *Drosophila* clot with fibers and incorporated plasmatocytes. (Courtesy of Ulrich Theopold, Stockholm University.) (*b*) Structure of Hemolectin. Hemolectin is a large protein with domains that are also present in other clotting factors (197). Hemolectin is a major component of *Drosophila* clotting fibers. This figure was done using the SMART bioinformatic tool (EMBL, Heidelberg) for identifications and positions of domains. (*c*) A model for clot formation at an injury site. Upon injury, plasmatocytes immediately release Hemolectin and other proteins that form clot fibers. Cross-linkage of these fibers occurs with the help of proteins such as Fondue, transglutaminase, and proPO, the latter being released by crystal cells.

Melanization

An immediate immune response in *Drosophila* is the melanization reaction observed at the site of cuticular injury or on the surface of parasites invading the hemocoel (**Figure 6**). This blackening reaction results from the de novo synthesis and deposition of melanin. It is generally assumed that melanization plays an important role in arthropod defense reactions such as wound healing, encapsulation, sequestration of microorganisms, and the production of toxic intermediates that are speculated to kill invading microorganisms (206–208). Melanization requires the activation of proPO, an enzyme that catalyzes the oxidation of mono- and diphenols to orthoquinones, which polymerize nonenzymatically to melanin. Enzymatically inactive proPO is cleaved into active phenoloxidase (PO) by an SP known as prophenoloxidase activating enzyme (PPAE). PPAE also exists as an inactive zymogen that is itself stimulated through a stepwise process involving other SPs. Studies with other insect species indicate that the melanization cascade is triggered by injury or by recognition through PRRs of microbial ligands, such as PGN, β(1,3)-glucan, and LPS (209–212). Consequently, the proPO cascade is an efficient nonself-recognition system in invertebrates.

PO: phenoloxidase

PPAE: prophenoloxidase activating enzyme

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The *Drosophila* genome encodes three proPOs: DoxA1, DoxA3, and *CG8193*. *DoxA1* and *CG8193* are expressed in crystal cells while *DoxA3* is exclusively expressed in lamellocytes and as a consequence may participate in melanization during encapsulation (186). Melanization at injury sites in larvae is mediated exclusively by crystal cells and is impaired in three classical hemocyte mutants: *domino*, which lacks hemocytes (213); *Black cells*, which has aberrant crystal cells; and *lozenge*, which lacks crystal cells (161). None of the classical immune pathways (Toll, Imd, JAK-STAT) is involved in the rapid release of PO by crystal cells, but the rupture of crystal cells and subsequent melanization are blocked when the function of the RhoA GTPase is altered (81). The source of PO in adults that are devoid of crystal cells is not known. Recently, two Clip domain-containing SPs, MP1 and the crystal cell-specific MP2/sp7/PAE, and one serpin, Serpin27A, have been implicated in the melanization cascade (143, 214–217). Inactivation of MP1 and MP2 reduces the level of PO activity after immune challenge, while excessive melanization is observed in Serpin27A-deficient mutants. The role of Serpin27A is to restrict melanization to the site of injury and prevent systemic melanization. These analyses also suggest that MP1 is involved in the defense against both bacteria and fungi, whereas MP2 is more specific for the antifungal response (217). Except for these reports, our information on the organization of proteolytic cascades that regulate proPO activation is still scarce, and a possible link with PRRs remains to be established.

DROSOPHILA IMMUNITY AND PATHOGEN EVASION STRATEGIES

Immune defense mechanisms are selected for their capacity to confer resistance to microorganisms and parasites encountered in the wild. Their organization somehow reflects the evolution of interactions between the host and its pathogens. It is therefore important to understand how an immune system works in response to its pathogens and the diseases they inflict on the host. A major difficulty for a detailed understanding of the fly immune system is our ignorance of the impact of various pathogens on *Drosophila* populations. This situation is complicated by the fact that *D. melanogaster* belongs to a fruit fly species that recently spread across the world and is often associated with human habitats. This broad ecological niche does not help to narrow down the list of natural *Drosophila* pathogens and their effects. However, a number of *Drosophila* pathogens have been identified in laboratories or in the wild (218), some of which are currently used to analyze the immune response under more physiological conditions.

In the second section of this review, we first describe various infection models used to study *Drosophila* immunity and then discuss the evasion strategies developed by pathogens to escape the immune response, thereby revealing the complexity of host pathogen interactions in an insect model.

Immunity to Bacteria

Immunity to septic injury. Our knowledge of the *Drosophila* immune response is essentially based on the analysis of host reactions following injection of nonpathogenic bacteria (e.g., *E. coli* or *Micrococcus luteus*) either by pricking the body wall with a sharp needle coated with bacteria or by the microinjection of a precise dose of microbes directly into the body cavity of the insect. As discussed above, directly introducing a microorganism into the hemocoel triggers a rapid response that allows monitoring of the multiple facets of the interactions between a microorganism and the systemic immune defense (219). The expression of AMP genes is usually proportional to the number of bacteria injected and reaches a plateau or even decreases when flies are injected with massive doses of bacteria (108). There is no equivalent of an overactive inflammatory response in adult *Drosophila* comparable to the septic shock in mammals, and

flies are resistant to injection of large amounts of bacteria such as *E. coli*.

Mutations affecting the Imd or the Toll pathways cause the most severe immune deficiency to bacterial challenge, and death is always associated with excessive bacterial growth (44). Death of the host usually occurs when numbers of bacteria increase beyond 10^6 per fly. Inhibition of phagocytosis through injection of Sephadex beads that saturate the plasmatocytes has no effect in wild-type adults but increases the susceptibility of *imd* mutant flies to *E. coli* (220). Similarly, an effect of melanization against bacteria can be detected when a mutation affecting this process is combined with Toll or Imd pathway mutations (55, 217). Survival experiments in larvae carrying combinations of mutations affecting hemocytes (*domino*), the antibacterial response (*imd*), and melanization (*Black cells*) after an immune challenge with *E. coli* establish the relevance of all three immune reactions in larvae—AMP production, phagocytosis, and melanization—and point to a synergy between them to fight infection (213).

***Two fast killers:* Pseudomonas aeruginosa *and* Serratia marcescens.** Unlike the situation described above, injection of a small number of cells of *Pseudomonas aeruginosa* or *Serratia marcescens* into the hemolymph is fatal to wild-type flies (7, 221–223). Both bacteria are opportunistic and versatile pathogens that have developed the ability to adapt to a large number of environmental conditions. The reasons why these two bacterial species are so pathogenic to flies are not clearly established. Following injection, *P. aeruginosa* invades and degrades fly tissues and uses them as a nutrient source (224). A prior injection with nonpathogenic variants partially protects against a subsequent infection by *P. aeruginosa*, and this effect is Toll- and Imd-dependent. Mutants deficient for Toll and Imd pathways are more susceptible to *P. aeruginosa* than are wild-type flies (224, 225). This indicates that *P. aeruginosa* is somewhat sensitive to the fly humoral response, but can ultimately overcome it. A microarray analysis indicated that AMP gene expression is delayed when flies are challenged with a pathogenic strain of *P. aeruginosa* as compared to nonpathogenic bacteria (225). Altogether these data point to the existence of a complex set of strategies used by *P. aeruginosa* to suppress and escape the immune response.

Many *P. aeruginosa* mutants with decreased pathogenicity have been identified, but none of these mutations leads to a complete loss of virulence (219, 221, 226). Toxins such as phenazine and pyoverdine, as well as injection of virulence factors into host cells through the Type III secretion system, participate in virulence. A recent study indicates that *P. aeruginosa* suppresses its own phagocytosis by *Drosophila* hemocytes through the injection, via the Type III apparatus, of a Rho-GAP GTPase toxin that impairs hemocyte function (227, 228).

***Two slow killers:* Mycobacterium marinum *and* Salmonella typhimurium.** *Mycobacterium marinum*, a pathogenic bacterium closely related to *M. tuberculosis*, causes a tuberculosis-like disease in fish and frogs. It is lethal to flies within 8 days following injection of a low dose (229). *M. marinum* initially proliferates inside plasmatocytes and spreads later in the course of infection to the whole organism. Intracellular *M. marinum* blocks vacuolar acidification, suggesting that it successfully subverts fly plasmatocytes as it does with vertebrate macrophages. Consistent with an intracellular localization of this bacterium, injection of *M. marinum* does not trigger AMP gene expression and as a consequence *imd* and *Toll* mutant flies do not exhibit higher susceptibility than wild-type flies. Flies infected with *M. marinum* progressively lose energy reserves, and this wasting-like process participates in the pathogenesis (229a).

Similarly, injection of *Salmonella typhimurium* causes death within 7–9 days. Bacterial growth in the hemolymph is limited by the Imd pathway (230). Like *M. marinum*,

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this bacterium survives in hemocytes and impairs their function, leading to slow death.

Ecc15: *Erwinia carotovora* 15

Although *M. marinum* and *S. typhimurium* are not true *Drosophila* pathogens, these studies suggest that one mechanism used by bacteria to resist the systemic immune response is to reside inside hemocytes and to alter their phagocytic function, similar to intracellular pathogens in vertebrates.

Drosophila *as a model host to identify bacterial virulence factors.* The good correlation between virulence factors of *P. aeruginosa* required to infect *Drosophila* and those required to infect vertebrates, as well as the observation that many human pathogenic bacteria have a broad host range including invertebrates, has launched the use of *D. melanogaster* and *C. elegans* as alternative model hosts (219, 231, 232). Indeed, several studies suggest that *Drosophila* can be used to screen for virulence factors and to analyze the complex interactions between pathogenic bacteria and the innate host defense (219, 221–230, 233–234). Recently, high-throughput RNAi screens in S2 cells have been developed to systematically screen for host components required for bacterial internalization and spreading. Such large-scale screens have been applied to *L. monocytogenes*, *Legionella pneumophila*, and *Mycobacterium fortuitum* (176–179, 233, 234).

Even though the requirement for some virulence factors depends on a specific host, invertebrate models may now provide a powerful tool to reveal new aspects of bacterial pathogenesis.

Immunity after oral bacterial infection. Injuries are probably not a frequent source of entry of pathogens in *Drosophila*, unlike oral infection upon ingestion. One limitation of approaches using injection is that they bypass the initial steps of naturally occurring infections including bacterial colonization, persistence, and the host local immune response. In addition, the microorganisms generally used are not true pathogens of *Drosophila*. To overcome this limitation, the use of natural oral infections with infectious bacteria has been developed.

***Flora and bacterial pathogens of* Drosophila.** *Drosophila* flies live on fermenting medium and as a consequence their gut flora will reflect bacteria living in their food (218). It is not clear whether a stable gut flora exists in this insect. The study of flies raised axenically (deprived of their normal flora) points to a contribution of this flora to the physiology of these insects (218). To date, the influence of this natural gut flora on the *Drosophila* immune system has not been investigated.

Fruit flies have been involved in the transmission of many phytopathogenic bacteria and probably participate in their dissemination in the environment (218). Recently, a few bacterial strains were found to be able to trigger an immune response in flies or to be pathogenic after oral infection. These include *S. marcescens* Db11, *Erwinia carotovora* 15 (Ecc15), and *Pseudomonas entomophila*. These bacteria can establish a persistent interaction with their host.

S. marcescens *Db11.* *S. marcescens* Db11 was isolated from moribund flies in the laboratory (222). Oral infection by *S. marcescens* kills flies within 4 to 8 days. This bacterium persists in the gut where it triggers local AMP gene expression and crosses the intestinal barrier to reach the hemocoel without, however, eliciting a systemic immune response (81). *S. marcescens* may not be detected by the fly immune system as a result of a low release of PGN into the hemolymph. Two host defense mechanisms contribute to host survival against *S. marcescens*: (*a*) local AMP expression in the gut and (*b*) phagocytosis of *S. marcescens* in the hemolymph (81, 171).

Erwinia carotovora 15 (Ecc15). A Gram-negative bacterial strain, *Ecc15*, was initially identified for its capacity to persist in the

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Drosophila gut and to trigger both local and systemic immune responses following oral infection (82, 154). Both responses are mediated by the Imd pathway. *E. carotovora* are phytopathogenic bacteria that cause soft rot in fruit and use insects as vectors. Ecc15 persists in the gut of larvae while not killing them. This effect is mediated by a single gene, *Erwinia virulence factor* (*Evf*) (235). *Evf* plays a direct role in gut persistence as its transfer into other Gram-negative bacteria enables them to persist in the *Drosophila* gut and to elicit a systemic immune response (236). *Evf* encodes a novel protein with no homology in the databases. The exact mechanism by which *Evf* promotes bacterial persistence in the gut is not known. That *Evf* is found only in a subset of *E. carotovora* strains that have infectious properties toward *Drosophila* suggests that it is an example of a gene that promotes survival and dissemination of bacteria in their environment.

P. entomophila. *P. entomophila* is a novel *Pseudomonas* species collected from a fly in Guadeloupe (237). In contrast to Ecc15, *P. entomophila* is highly pathogenic for *Drosophila* as well as for other insect species by destroying the gut. In addition, this bacterium induces a food-uptake blockage in *Drosophila* larvae. Although *P. entomophila* triggers both local and systemic activation of the Imd pathway, only the gut antimicrobial response contributes to host survival after oral infection (238). One mechanism used by *P. entomophila* to counteract the gut immune response is the secretion of an abundant protease, AprA, that degrades AMPs produced by gut epithelia and thereby promotes bacterial persistence (238). *S. marcescens* also expresses a protease (222). Taken together, these results suggest that proteases may represent a common strategy used by pathogens to circumvent the local antimicrobial host defense of insects (**Figure 5**). Many pseudomonads and other bacteria express proteases similar to AprA but are not able to infect *Drosophila* by oral ingestion. This indicates that *P. entomophila* virulence is multifactorial, AprA being one virulence factor among others. The complete sequencing of the *P. entomophila* genome has revealed the existence of large sets of genes encoding putative virulence factors such as proteases, lipases, toxins, and proteins involved in alginate synthesis (239).

Gut infection and fat body immune response. Both *Ecc15* and *P. entomophila* can trigger a strong systemic immune response in *Drosophila* after oral infection, pointing to the existence of a signaling mechanism from the gut to the fat body. This immune response correlates with the capacity of these bacterial species to persist and multiply inside the gut and does not appear to rely on physical crossing of the gut wall (236, 237). It has been proposed that this systemic immune response is mediated by the translocation of small PGN fragments from the gut lumen to the hemolymph (116). This view is supported by the observation that ingestion of monomeric PGN can stimulate a strong systemic immune response in PGRP-LB RNAi flies that have reduced amidase activity and are unable to degrade PGN to its nonimmunogenic form. Transfer of PGN would provide an indirect mechanism for recognition of Gram-negative bacteria that may explain the existence of different PGRP-LC isoforms devoted to the detection of monomeric PGN. Alternatively, one study points to a key role of nitric oxide as a signaling molecule, in that ingestion of nitric oxide is sufficient to trigger an immune response in the absence of an infection (240, 241).

Finally, a role for hemocytes in the signaling between gut infection and fat body has been proposed based on the observation that *domino* larvae lacking hemocytes failed to activate a systemic immune response after *Ecc15* infection (154). Cells that express hemocyte markers are embedded in the first loop of the midgut (B. Lemaitre, unpublished data). These *Drosophila* hemocytes might act similarly to vertebrate M-cells that have an immune function in the vertebrate digestive

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tract by sampling the contents of the gut lumen.

Natural bacterial infection: concluding remarks. The use of naturally infectious bacteria has revealed the important contribution of the local immune defense (81, 158, 238). From this point of view, note that local AMP synthesis in epithelia is a conserved defense mechanism within the animal kingdom, whereas systemic AMP production by the fat body is probably a more recent adaptation because it appears to be specific to holometabolous insects. Natural infection studies also reveal common strategies used by bacteria to subvert the insect immune system such as the production of proteases. Why a given bacterial species can trigger an immune response upon ingestion is not yet understood. This could be a simple consequence of bacterial persistence in a compartment of the gut (leading to a local increase of a bacterial elicitor) or could reflect specific properties of the bacteria (release of PGN). Furthermore, *P. entomophila* infection triggers a systemic immune response that has no overt function against bacteria remaining in the gut lumen.

Bacterial endosymbionts: *Wolbachia*. In contrast to other insect groups, heritable symbionts are uncommon in *Drosophila* species, possibly reflecting a robust innate immune response that eliminates many bacteria (218). The most frequent endosymbionts are *Wolbachia*, which are intracellular bacteria present in 30% of all laboratory stocks as well as in populations in the wild. *Wolbachia* parasites, which can only be transmitted vertically from a female host to her offspring, have evolved strategies for maximizing their transmission, such as changing the sex of male fly embryos. They colonize many different tissues but have a specific tropism to reproductive tissues to ensure their transmission. The recent discovery of PGRP-LE as an intracellular sensor of Gram-negative bacteria opens the possibilities of an immune defense to intracellular bacteria such as *Wolbachia* (127).

Immunity to Fungi

Extensive ecological studies have been performed on the association between flies and yeasts. In the wild, flies also play a role in the dissemination of phytopathogenic fungi. To date, the fly immune response has been analyzed in detail in response to two fungi: *Beauveria bassiana* and *Candida albicans*.

B. bassiana. *B. bassiana* is an entomopathogenic fungus that infects many insect species by penetrating their cuticle. Conidia of *B. bassiana* germinate on the host cuticle and differentiate to form an appressorium. An infecting hypha penetrates through the host cuticle and eventually reaches the hemocoel. Studies on *Metarhizium anisopliae*, a fungus related to *B. bassiana*, have shown that entry into the host involves enzymatic degradation mediated by chitinases, lipases, and proteases such as PR1 (242). Natural infection of *Drosophila* by *B. bassiana* (initiated by applying fungal spores on the cuticle) leads to the expression of the antifungal peptide genes *Drosomycin* and *Metchnikowin* and other Toll-dependent immune genes through a selective activation of the Toll pathway (19, 83). *Toll*-deficient mutants show increased susceptibility to *B. bassiana*, which cannot be rescued by ectopic expression of *Drosomycin* (18). Thus, the mechanisms mediating Toll resistance against *B. bassiana* have yet to be established. Finally, melanization is also believed to play an important role in the insect host defense mechanism against fungi (214, 217).

Activation of the Toll pathway by fungi is mediated by GNBP3 via the sensing of β(1,3)-glucan. Curiously, even *GNBP3* mutant flies upregulate their Toll pathway when they become infected with *B. bassiana* (131c). In this condition, the Toll pathway is thought to be triggered by a fungal protease PR1, which can lead to cleavage of the host SP Persephone. In a second step, Persephone activates the SP cascade acting upstream of Toll. Thus, flies may sense infection by *B. bassiana* through

the production of a fungal virulence factor required for entry.

C. albicans. *C. albicans* is an opportunistic human pathogen. It is a dimorphic fungus, and it has been suggested that the yeast-to-hyphae transition plays a pivotal role in its virulence. Injection of *C. albicans* into wild-type flies induces moderate killing, but *Toll*-deficient mutants are highly sensitive to this fungus (243). *C. albicans* proliferates extensively as pseudohyphae, in a manner similar to that which occurs in a mammalian host model. Toll pathway activation by *C. albicans* requires GNBP3 (131c). In addition, *C. albicans* is rapidly phagocytosed by hemocytes, and experiments in S2 cells have shown that this process is enhanced by Mcr (178).

Immunity to Parasitoid Wasps

Parasitoids are insects, mostly wasps (Hymenoptera), that parasitize invertebrates, mainly insects (244). In contrast to parasites, they ultimately kill their hosts by the ravenous feeding of the preimaginal stages of the parasitoids. Depending on the species, females lay one or more eggs into their host. Upon completion of their development, adult parasitoids emerge, mate, and disperse in the environment in search of hosts to complete their life cycle (**Figure 8*a***). Of the approximately 50 known species of *Drosophila* parasitoids, those belonging to the *Leptopilina*, *Asobara*, and *Ganapsis* genera have been studied most intensively. Environmental studies indicate that there is a high degree of parasitization of *Drosophila* populations by wasps (244). More recently, immunological aspects of *Drosophila*-parasitoid relationships have been analyzed. In *Drosophila*, cellular encapsulation is the most important component of the immune response against parasitoids (**Figure 8*b*,*c***). *D. melanogaster* and *Drosophila yakuba* resistance to infestations of *Leptopilina boulardi* are both under the genetic control of one major gene (245, 245a). For successful parasitism, parasitoids must circumvent the host immunity using passive and/or active mechanisms.

Immune local evasion. Immune local evasion is achieved when the parasitoid egg develops in a host site inaccessible to hemocytes or is embedded into host tissues. The egg can also use a type of molecular disguise that prevents hosts from recognizing it as nonself or can be covered by factors causing a local inactivation of the host defense. An example of local evasion is observed in *Asobara tabida*, a braconid wasp that parasitizes *Drosophila* larvae in Europe and North America (246, 247). Parasitization by *A. tabida* provokes a transient activation of the host immune system. However, eggs of *A. tabida* possess a sticky chorion permitting their attachment to host tissues. Attachment of the chorion leads to the complete embedding of the parasitoid egg within the host tissues, preventing hemocytes from agglutinating at the egg surface. Thus *A. tabida* eggs are initially recognized by the host, but there seems to be a race between the immune reaction and the parasitoid evasion mechanism to avoid encapsulation (248). The molecular basis of this evasion mechanism is not known.

Active immune suppression. The active suppression of the immune response generally involves substances that are introduced into the host by the female wasp at the time of oviposition. These mechanisms involve female products such as ovarian protein venoms, virus-like particles, or polydnaviruses. One of the best examples of active immune suppression is observed in the interaction between *D. melanogaster* and the parasitic wasps *L. boulardi* or *L. heterotoma*. Most strains of these wasp species are able to bypass the immune response of *D. melanogaster*. They suppress the fly encapsulation response notably by changing lamellocyte morphology from a discoidal to a bipolar shape, which presumably diminishes their ability to adhere and to form capsules (**Figure 8*d***) (249–251).

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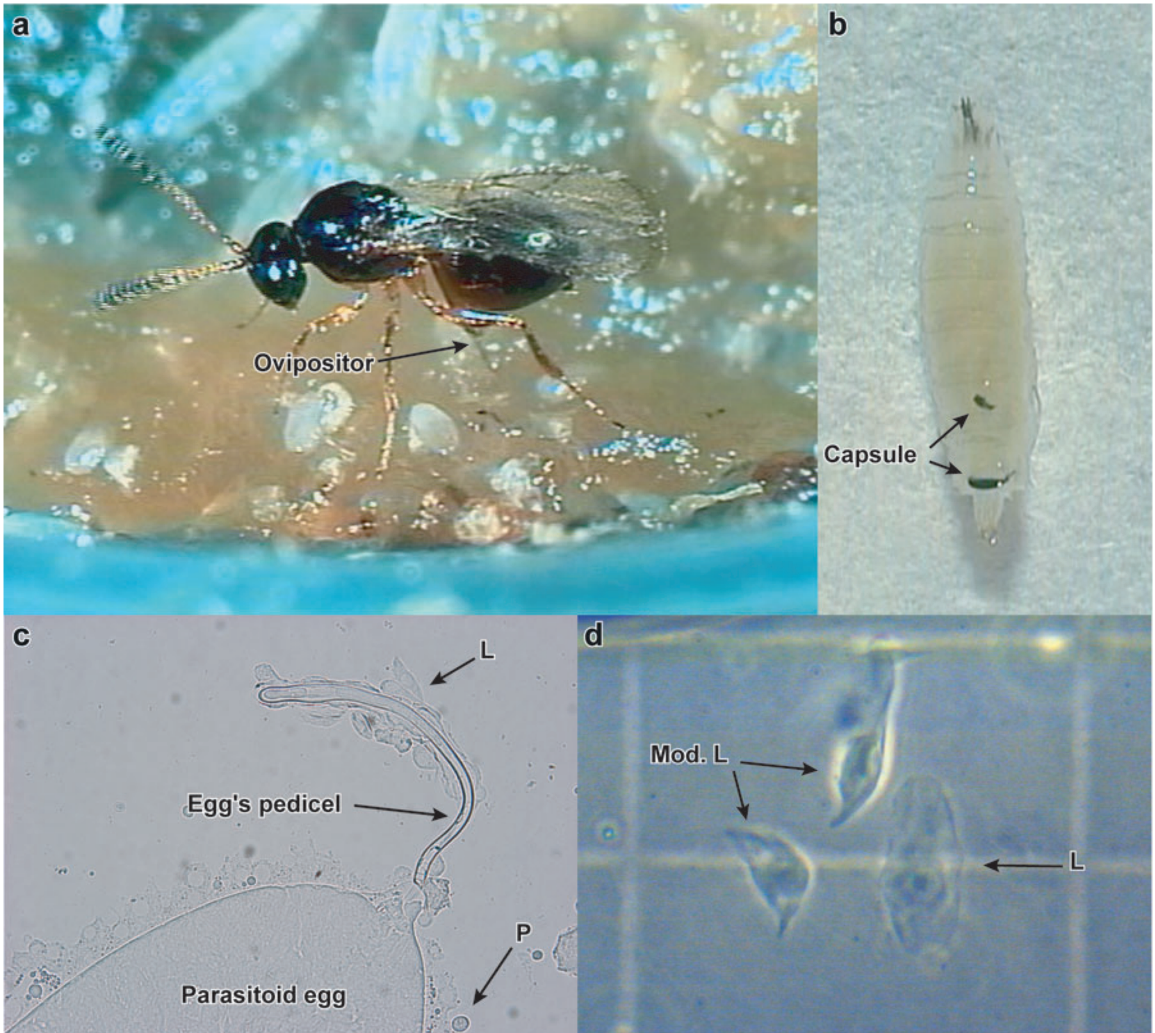

Figure 8

Drosophila infection by parasitoid wasp. (*a*) The parasitic wasp *Leptopilina boulardi* infects *Drosophila* second instar larvae (L2) and deposits eggs within the body cavity. (*b*, *c*) Encapsulation of the egg is the major host defense against parasitoid infection. The black capsules are visible through the larval cuticle (*b*). P, plasmatocyte. (*d*) *L. boulardi* can suppress the *Drosophila* encapsulation response by altering lamellocyte (L) morphology from a discoidal to a bipolar shape. L, normal lamellocyte; mod. L, bipolar-shaped lamellocyte. Courtesy of Aurore Dubuffet (*a–c*) and Corinne Labrosse (*d*), University of Tours, France.

Virus-like particles (VLPs) produced in the female long gland and the reservoir are injected together with the eggs and have been associated with immune suppression. P4, one of the proteins present in the long glands, was recently isolated from *L. boulardi* and demonstrated to be a Rho-GAP domain-containing protein (252, 253). Rho-GAP proteins are acting as inhibitors of Rho GTPases that are involved in the actin cytoskeleton reorganization. The observations that (*a*) the ability to detect the P4 protein in female parasitoids correlates with their virulence and (*b*) its injection in the host alters lamellocyte morphology

establish this protein as a main immune-suppressive factor of *L. boulardi*. Similarly, a 40-kDa protein with immune-suppressive properties was identified at the surface of VLPs in *L. heterotoma* (254, 255).

Another strategy of immune suppression is observed with *Asobara citri*, a parasitoid devoid of VLPs. *A. citri* appears to suppress the host immune system by directly affecting the hematopoietic organ and, consequently, the number of circulating hemocytes (256). Indeed, the size of the anterior lymph glands is strongly reduced in larvae of *D. melanogaster* parasitized by *A. citri*. The molecular mechanism underlying lymph gland necrosis caused by *A. citri* is not known.

ANTIVIRAL IMMUNITY

Viruses are important natural pathogens of *D. melanogaster*: 40% of all flies are infected with horizontally transmitted viruses, and vertical transmission of viruses is also common in this species (218, 257). More than 25 distinct *Drosophila* viruses have been identified, all RNA viruses (218, 257a). Although there is an extensive literature on the association between viruses and *Drosophila*, only the immune response to viruses, especially to the Sigma and *Drosophila* C virus (DCV), has been investigated. *Drosophila* host reactions to viruses are clearly distinct from those involved in the defense against microbes or parasites. To date, RNAi is the sole immune effector mechanism directed against viruses identified in this insect.

Sigma Virus

The Sigma virus, a rhabdovirus related to vesicular stomatitis virus of horses, is widespread in natural populations of *D. melanogaster* and is normally transmitted vertically from fly to fly via the egg. Viruses can, however, also be artificially transmitted by injection of tissue extracts derived from an infected fly. Flies carrying the Sigma virus appear normal, but die after benign exposure to carbon dioxide. Natural populations of *D. melanogaster* are often polymorphic for nonpermissive alleles of genes that are known collectively as *ref* (*refractory*) and that prevent virus replication in cells. *Ref(2)P*, the best-characterized among the five *ref* genes identified, encodes a PEST domain protein that is highly variable in sequence between alleles of natural populations (258, 259). A null mutation in *ref(2)P* is permissive to the Sigma virus, but causes male sterility, indicating that this gene has an endogenous function in flies. The molecular mechanism by which the restrictive allele of *ref(2)P* blocks Sigma replication is not known, although direct interaction has been suggested between *ref(2)P* protein encoded by restrictive alleles and the viral RNA polymerase (260). Because the Sigma virus can mutate to overcome the fly resistance to infection resulting from a refractory phenotype, there is probably an arms race between the viral genome and the host genome. Collectively, these studies indicate that a high polymorphism of *ref* genes plays an important role at the level of populations to limit Sigma infections.

DCV: *Drosophila* C virus

Drosophila C Virus (DCV)

DCV is a nonenveloped single-stranded RNA virus similar to vertebrate picornaviruses. Flies isolated in different parts of the world display varying susceptibility to this virus. It is horizontally transmitted through contact or ingestion, but to date only injections have been used to ensure reproducible infection and lethality under laboratory conditions. Genome screening with S2 cells has identified a large number of *Drosophila* genes required for virus entry and replication, notably genes encoding components of the clathrin-mediated endocytic pathway (261–262a).

Proteomic and microarray analyses were performed on DCV-injected flies to analyze the global immune response to DCV. One molecule, *pherokine-2*, was found to be induced at the posttranscriptional level in the

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hemolymph of DCV-infected flies (263). The high expression of *pherokine-2* during metamorphosis pointed to a possible role of this gene in tissue remodeling. DCV infection triggers a transcriptional response in flies that significantly differs from that triggered by bacteria or fungi (91). Some of the DCV-specific responsive genes are regulated by the JAK-STAT pathway, a cascade that plays an important role in the antiviral response in mammals. One of the DCV-responsive genes, *vir1*, is induced 24 h after viral infection in the ventral epidermis, but not in the fat body, in a JAK-STAT-dependent manner. A role for the JAK-STAT pathway in antiviral defense is also supported by the observation that flies deficient in the JAK kinase Hopscotch exhibit increased susceptibility to DCV and contain a higher viral load. These data suggest that flies produce antiviral molecules in a JAK-STAT-dependent manner, although, to date, none of the identified DCV-regulated genes has been shown to manifest direct antiviral activity. DCV infection does not induce expression of the JAK-STAT-dependent stress-induced gene *Turandots*, at least in the first 3 days of infection before tissue damage becomes apparent. Some components of the Toll pathway (Toll and Dif, but not Pelle and Tube) have also been implicated in the resistance against *Drosophila* X virus (264).

These studies support the existence of an antiviral signaling response in *Drosophila* and raise many new questions (265). For one, the mechanisms by which *Drosophila* recognizes viral infection have not yet been worked out. In particular, it will be important to determine whether the transcriptional response to viral infection is triggered by intracellular pattern recognition receptors and/or can depend on signals from virus-induced tissue damage. A second point of importance will be the identification of antiviral molecules in *Drosophila*. Finally, it seems appropriate to study the immune response after natural viral infection because the route of entry may influence the host response: Only few genes were induced in microarray studies after oral ingestion of DCV (266), in contrast to the robust response observed after DCV injection (91).

RNAi as an Antiviral Immune Response Against RNA Viruses

RNAi provides an antiviral defense in plants and animals that is able to seek out and destroy viral RNA. RNAi is initiated by the recognition of dsRNA through Dicer 2, which cleaves newly synthesized viral dsRNA, generating siRNAs (small interfering RNA) that "guide" the RISC complex to specifically recognize and degrade viral RNA. Recent reports have underlined the importance of this mechanism in the *Drosophila* antiviral response. Mutations in *argonaute-2* or *Dicer 2*, which affect the RNAi pathway, increased the susceptibility of flies to a large number of RNA viruses including DCV, Flock House virus (FHV), Sinbis, and *Drosophila* X virus (267–269a). Successful infection and killing of *Drosophila* by the insect nodavirus or FHV are strictly dependent on the viral protein B2, a potent inhibitor of dsRNA processing by Dicer. A significant role of RNAi in the *Drosophila* antiviral defense is further supported by the observation that three genes involved in the RNAi response against viruses, but not their housekeeping miRNA paralogs, are in the top 3% of fast-evolving genes in the *D. melanogaster* genome, which is often a signature of a host-pathogen arms race (270).

Although still preliminary, these studies suggest the existence of two types of responses to virus infection: degradation of viral RNA by RNAi and cytokine activation through the JAK-STAT pathway of a number of genes, which may counter viral infection.

PERSPECTIVES

In recent years considerable progress has been made in our understanding of the immune response of *Drosophila*, now one of the best-characterized host defense systems of metazoans. The success of this model largely

relies on the possibility of applying genetic and genomic approaches to this organism. This situation will probably prevail in the future because *Drosophila* genetics remain among the most powerful and cost-effective approaches when compared to other models. Indeed, injection of dsRNA, which opened the route to functional studies in other insects, has its limitations. In particular, the injection procedure can perturb the response under investigation, and this may be critical when analyzing subtle aspects of the immune system. Ultimately, classical genetics, including the generation of animals carrying multiple mutations in defined genetic backgrounds, are feasible in *Drosophila*, but not in other insects. Such methodology thus remains mandatory to the generation and validation of solid data.

An attractive feature of the fly immune response is the existence of multiple defense "modules" that can be deployed in a coordinated response against distinct pathogens. The prominent immune reactions, such as AMP production, iron sequestration, phagocytosis, and ROS production, appear to be shared with vertebrates, whereas others, such as melanization and encapsulation by lamellocytes, are restricted to invertebrates. Analyzing the precise molecular mechanisms underlying each of these reactions remains an essential task for our full understanding of the fly immune response. In parallel, investigations concentrating on the global immune response to one specific pathogen are complementary since they provide a more integrated view of the immune system demonstrating how these defense "modules" interact. The hope is that these combined approaches will reveal more coherence among the many aspects of fly defense mechanisms, which still appear dissociated. In the future, addressing the interactions between immunity and other physiological functions (e.g., metabolism, or adaptation to environmental stress) will assist in discerning the relationships of the immune system with other aspects of fly life.

The remarkable similarities between *Drosophila* host defense and essential facets of vertebrate innate immunity clearly point to a common ancestry in evolution. This is particularly striking with regard to the intracellular signaling pathways activated upon infections. A precise understanding of the evolution of innate immune responses in metazoans will obviously require the study of these defense reactions in other invertebrate species and should allow discrimination between recent acquisitions and conserved mechanisms. From this point of view, the recent genomic sequencing of several *Drosophila* species as well as that of other insects has also launched *Drosophila* as a model for studying microevolution between closely related species. Several interesting studies, not discussed in this review, are investigating the genetic basis of immune competence variations among *Drosophila* populations and the diversity of immune genes among insect species. Major differences can be observed between closely related species. To illustrate this, some *Drosophila* species, such as *D. pseudoobscura*, lack the capacity to encapsulate parasitoids due to the absence of lamellocytes (183). It has been proposed that this natural case of constitutive immune deficiency could represent an unexpected and risky strategy: The absence of a particular immune "module" may be beneficial as it economizes valuable resources allocated to this mechanism. Thus, studies comparing immune systems of related species or even populations of *D. melanogaster* may reveal differences in host defense strategies. This comparison will probably underline the importance of ecology in shaping an immune system and should lead to a better understanding of adaptation. As Jiggins & Kim point out (271), there is no reported specialist fungal or bacterial pathogen for *D. melanogaster*. This may be related to its ecology as *Drosophila* have short life spans and live on ephemeral food patches. It is possible that most *D. melanogaster* pathogens are generalists infecting many species, which in

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turn restricts the opportunity for coevolution. This mode of life has strong implications for determining the nature of the immune system. Future studies may well reveal interesting links between *Drosophila* immune defense and the ecology of this insect.

ACKNOWLEDGMENTS

The authors apologize for their inability to quote many additional contributions due to space limitations. We thank Charles Hetru for graphics conception; Christoph Scherfer, Marie Meister, Anna Zaidman, and Marylène Poirié for critical reading; and Ulrich Theopold, Corinne Labrosse, and Aurore Dubuffet for providing images.

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