

14 Insect Immunology

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14.1. Introduction	480
14.2. Insect Immunology Background	480
14.2.1. Pioneering Studies of Insect Immunology	480
14.2.2. Basic Concept and Development of Innate Immunity	481
14.3. PAMP-Recognition Proteins in Insect Immunology	481
14.3.1. Peptidoglycan Recognition Proteins	481
14.3.2. β -1,3-Glucan Recognition Proteins	482
14.3.3. Lipopolysaccharide Recognition Proteins	483
14.4. Humoral Innate Immune Responses	483
14.4.1. Biochemical Properties of Insect Antimicrobial Peptides	483
14.4.2. Toll and IMD Signaling Pathways after Bacterial or Fungal Infection	485
14.4.3. Lectin Induction	490
14.4.4. Melanin Synthesis	491
14.4.5. Immune Responses to Viral Infection	494
14.4.6. Immune Responses to Malaria Infection	495
14.5. Cellular Innate Immune Responses	497
14.5.1. Phagocytosis	497
14.5.2. Hemocyte Hematopoiesis during Wasp Parasitism	499
14.6. Newly Emerging Topics in Insect Immunology	500
14.6.1. Gut Insect Immunology	500
14.6.2. Immune Priming of Insect Immunology	502
14.6.3. Hemimetabolous Insect Immunology	503
14.7. Conclusion	504

14.1. Introduction

Although insects do not have the adaptive immunity that defines the antigen-specific immune responses of vertebrates, insects do have innate immunity, which is comprised of cellular and humoral immune responses. The cellular innate immune responses, including phagocytosis, nodulation, and encapsulation, are mediated by hemocytes (insect blood cells). Clotting, melanin synthesis, and antimicrobial peptide (AMP) production, known collectively as humoral innate immunity, are mediated by soluble plasma proteins or fat bodies (corresponding to mammalian liver). Recent accumulated experimental data from insects, which are infected with pathogenic bacteria, fungi, parasites or viruses that specifically elicit their innate immune responses, provide strong clues that insects are useful model systems to study the innate immunity of invertebrates. As a result of drastic new technique developments in molecular cellular biology and genetics in the fruit fly, mosquito, and other large insects over the past 20 years, a dramatic explosion of knowledge of insect immunology has enabled us to understand its basic mechanisms. In this chapter, we will overview recent progress

in elucidating these molecular mechanisms, and current knowledge about the biological significance of insect immunology.

14.2. Insect Immunology Background

14.2.1. Pioneering Studies of Insect Immunology

In 1884, Elie Metchnikoff, a Russian scientist, introduced the word “phagocyte” as a name for the invertebrate circulating cells responsible for ingesting foreign bodies, and first discovered the phagocytotic immune response in insects. Another Russian scientist, Serge Metalnikov, intensively explored the mechanism of phagocytosis in insects. He began by describing the various types of insect hemocytes and their roles in phagocytosis and nodule formation. He found granular hemocytes (granulocytes) to be the major phagocytic cells. Metalnikov spent nearly three decades studying the phagocytosis of bacteria in two species of lepidopteran larvae: *Galleria mellonella* and *Pyrausta nubilalis*. His numerous observations led him to believe that phagocytosis was not one type of response but an extremely complex

phenomenon with degrees of intensity. Our molecular understanding of the cellular innate reaction in insects has advanced dramatically during the past two decades. Several receptors involved in phagocytosis and its regulatory mechanisms have recently been determined using genetic and molecular biology techniques (Stuart and Ezekowitz, 2008). Several molecules related to encapsulation have also been identified in large insect and mosquito systems.

In 1918, Rudolf W. Glaser first reported the existence of immunity in the grasshopper (Glaser, 1918). Next, June M. Stevens reported the identification of inducible bactericidal activity in the hemolymph (insect blood) of the wax moth *G. mellonella* (Stevens, 1962). True humoral insect immunology research was started by the Hans Boman group at Stockholm University, in an attempt to answer the question of how holometabolous insects survive against pathogenic microbial infection if their circulating hemolymph contains no antibodies and no immune cells capable of mounting an adaptive immune response. By injecting several different Gram-positive bacteria, they demonstrated that the fruit fly *Drosophila melanogaster* also had an inducible bactericidal effect in hemolymph (Boman *et al.*, 1972). The group realized that the purification of these bactericidal molecules from *Drosophila* would be exceedingly difficult, and Boman's colleagues turned instead to the giant silkworm *Hyalophora cecropia*, from which about 1 ml of hemolymph could be collected per pupa. After accumulating knowledge about inducible bactericidal effects, they finally reported the amino acid sequences of two novel antibacterial peptides called cecropins that are involved in insect innate immunity (Steiner *et al.*, 1981). This seminal discovery opened up a whole new field of insect immunology, leading to the isolation of AMPs from many different insects. Also, α -defensins were isolated from mammalian neutrophils only a few years after the identification and sequencing of cecropins (Selsted *et al.*, 1985), leading to the identification of a large number of AMPs found in vertebrates, and providing the essential knowledge for understanding immune defense in mammalian hosts (Zasloff, 2002). A variety of additional advances over the next 25 years resulted in the discovery of *Drosophila* Toll and IMD signaling pathways (Lemaitre *et al.*, 1996; Choe *et al.*, 2002; Gottar *et al.*, 2002), and human Toll-like receptors and their signaling pathways (Kawai and Akira, 2010).

14.2.2. Basic Concept and Development of Innate Immunity

In 1989, Charles A. Janeway first proposed the basic concept of innate immunity as the first-line host defense responses that serve to limit infection in the early hours after exposure to microorganisms (Janeway, 1989). He suggested that this system was activated by a group of germ-line-encoded receptors and soluble proteins, termed

pattern-recognition receptors and proteins, respectively. These pattern-recognition molecules were suggested to recognize microbial cell wall components that are conserved among microbes but absent in the host. These conserved motifs, called pathogen-associated molecular patterns (PAMPs), include lipopolysaccharide (LPS) of Gram-negative bacteria, peptidoglycan (PGN) of Gram-positive bacteria, and β -1,3-glucan of fungi (Medzhitov and Janeway, 2002). Upon recognition, these pattern-recognition receptors activate distinct signaling cascades leading to the expression of genes that participate in innate immune response, such as inflammatory cytokines or AMPs. Janeway also suggested that pattern recognition might be critically important not only in the early phase of infection but also in the initiation of adaptive immunity in vertebrates. His hypothesis has been proved by recent innate immunity studies in invertebrates and vertebrates. In 1996, Hoffmann and his colleagues reported that *Drosophila* Toll receptor controls the antifungal immune response in the fruit fly, and that mutations in the Toll signaling pathway dramatically reduce survival after fungal infection (Lemaitre *et al.*, 1996). In 1997, Janeway and his colleagues first identified a human homolog of the *Drosophila* Toll protein, and proved that this Toll-like receptor induced activation of adaptive immunity signals (Medzhitov *et al.*, 1997). This report opened up new research in mammalian innate immunity. Dramatic developments in the field of vertebrate and invertebrate innate immunity have contributed invaluable knowledge toward new drug development against infectious, inflammatory, and immune diseases that seriously affect modern human society (Hennessy *et al.*, 2010).

14.3. PAMP-Recognition Proteins in Insect Immunology

14.3.1. Peptidoglycan Recognition Proteins

PGN is a polymer consisting of glycan strands of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) that are cross-linked to each other by short-peptide bridges (Schleifer and Kandler, 1972). PGNs from Gram-negative bacteria and some *Bacillus* species differ from other Gram-positive PGNs by the replacement of lysine (Lys) residue with *meso*-diaminopimelic acid (DAP) at the third amino acid in the stem-peptide chain (Figure 1). Insect PGN recognition receptors (PGRPs) were first characterized in the *Bombyx mori* moth (Yoshida *et al.*, 1996), and proposed to be a trigger of the innate immune response in the moth. At present, many PGRPs have been purified or identified from different vertebrates and invertebrates, including *Drosophila* (Kang *et al.*, 1998; Royet and Dziarski, 2007). All PGRPs have so-called PGRP domains of about 160 amino acid residues that show high similarity to those of the bacteriophage

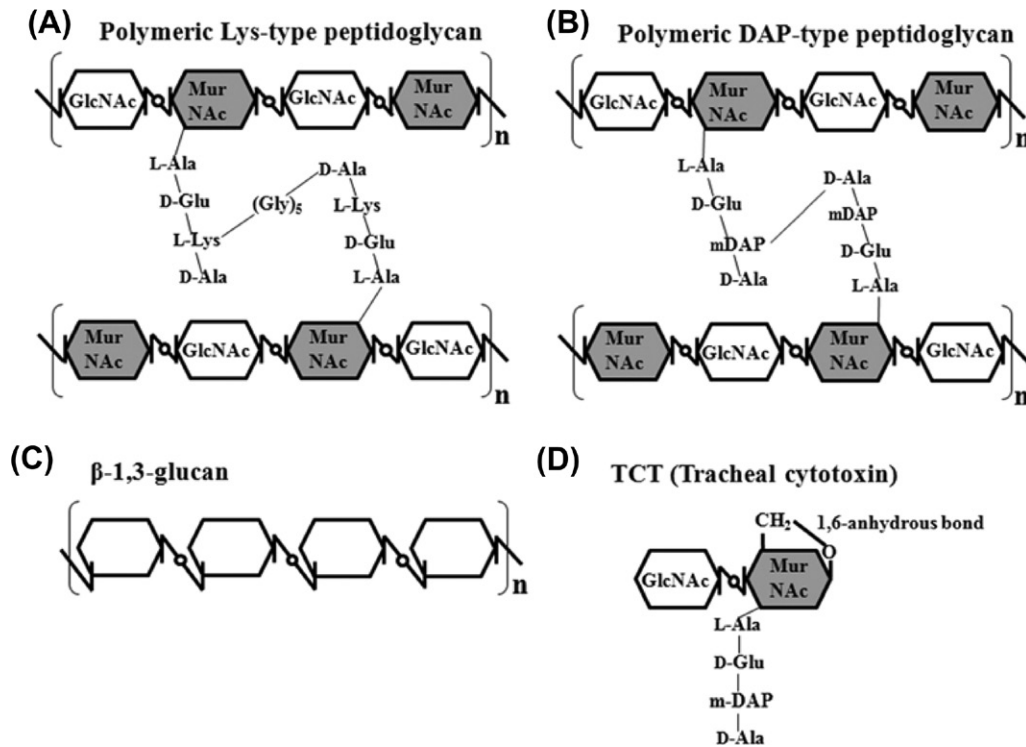


Figure 1 The primary structures of peptidoglycan, TCT and β -1,3-glucan. Peptidoglycan is a stem-peptide cross-linked polymer consisting of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). (A) Structure of *S. aureus* polymeric Lys-type peptidoglycan. The third residue of the stem-peptide is L-Lys. (B) Polymeric DAP-type peptidoglycan structure of *E. coli* containing a meso-diaminopimelic acid (DAP) residue at the third residue of the stem-peptide. (C) A polymer structure of β -1,3-glucan of yeast and some fungi. (D) Structure of monomeric DAP-type peptidoglycan containing an internal 1,6-anhydro bond at the MurNAc residue functioning as a ligand molecule of the IMD pathway. These molecules function as pathogen-associated molecular patterns (PAMPs).

T7 lysozyme, which is known to have a typical zinc-dependent N-acetylmuramoyl-L-alanine amidase. This amidase can cleave the amide bond connecting the stem-peptide to the carbohydrate backbone of PGN. The tertiary structures of several PGRPs, such as PGRP-LB, PGRP-LE, or PGRP-LC, show an extended surface groove domain that may be involved in the recognition of downstream molecules during the transfer of PGN signals, and in the interaction between the stem-peptide of PGN and the PGRP domain (Royet and Dziarski, 2007).

Drosophila has 13 PGRP genes that encode 17 PGRP proteins through alternative splicing. *Drosophila* PGRPs are classified into catalytic and non-catalytic PGRPs. PGRP-LB, -SB, and -SC belong to the catalytic PGRPs. Members of the second group, such as PGRP-SA, -SD, and -LC, lack the zinc-binding residues required for amidase activity, but still retain the ability to bind and recognize PGN. *Drosophila* PGRPs can also be categorized based on their size. The short-form proteins PGRP-SA, -SB, -SC, and -SD are secreted hemolymph proteins with PGRP domains and signal sequences, while the long-form proteins PGRP-LA, -LB, -LC, -LD, and -LE contain PGRP domains, signal sequences, and additional domains. PGRP-LA, -LC and -LD are membrane-associated

proteins, while others are assumed to be cytosolic (Werner *et al.*, 2000). Recent *Drosophila* genetic studies have demonstrated that two non-catalytic short-form PGRPs, -SA and -SD, are involved in the recognition of Lys-type-PGN and in the activation of the Toll signaling pathway. Two long-form receptors, PGRP-LC and PGRP-LE, are necessary for the recognition of DAP-type PGN and for the activation of the IMD signaling pathway (Lemaitre and Hoffmann, 2007) (see section 14.4.2).

14.3.2. β -1,3-Glucan Recognition Proteins

The β -1,3-glucans are polysaccharides of D-glucose monomers linked by β -1,3-glycosidic bonds that are important cell wall components of baker's yeast, mushrooms, and other fungi (Figure 1C). The first β -1,3-glucan recognition protein (β GRP) was purified from the hemolymph of the silkworm *B. mori* as a 50-kDa protein with a strong affinity for the cell wall of Gram-negative bacteria. The cDNA encoding this protein was isolated from an immunized silkworm fat body cDNA library, sequenced, and named the Gram-negative bacteria-binding protein (GNBP) (Lee *et al.*, 1996). Unexpectedly, the amino acid sequence of this GNBP protein contained a region with

significant homology to the putative catalytic region of a group of bacterial β -1,3-glucanases and β -1,4-glucanases. At the time, it was unknown whether this GGBP could bind to β -1,3-glucans. In 2010, the Ashida group determined the cDNA of the real β GRP, which has an ability to activate the β -1,3-glucan-mediated silkworm prophenol-oxidase cascade (Ochiai and Ashida, 2000). The deduced amino acid sequence of β GRP was not coincident with that of GGBP even though they were purified from the same insect, suggesting that *Bombyx* GGBP and β GRP may recognize different ligands and may regulate different immune responses in the silkworm. In 2003, the Hoffman group published a series of breakthrough *Drosophila* genetic studies. The *Drosophila* genome encodes three GGBPs (GNBP-1, -2, and -3) and two related proteins. Gobert *et al.* (2003) reported that *Drosophila* GNBP-1 functions as an essential molecule for the activation of the Gram-positive-mediated Toll signaling pathway. Furthermore, in 2006, Gottar and colleagues demonstrated that *Drosophila* GNBP-3 is involved in sensing fungal β -1,3-glucan and functions as an upstream β -1,3-glucan recognition receptor of the Toll pathway (Gottar *et al.*, 2006). The exact biological function of *Drosophila* GNBP-2 has not yet been determined. Based on these pioneer works, *Drosophila* GNBP-1 and GNBP-3 homologs have been identified from several different insects, such as *Anopheles gambiae*, *Manduca sexta*, and *Tenebrio molitor*, and their biological functions characterized (see below). Recently, the tertiary structures of the N-terminal domain of *Drosophila* GNBP-3 have been solved, showing that this domain contains an immunoglobulin-like fold in which the glucan-binding site is masked by a loop that is highly conserved among glucan-binding proteins (Mishima *et al.*, 2009).

14.3.3. Lipopolysaccharide Recognition Proteins

Hemolin, identified as a bacteria-inducible immune gene in the giant silkworm *H. cecropia*, is a member of the immunoglobulin gene superfamily comprised of four immunoglobulin domains (Sun *et al.*, 1990). When bacteria were injected into the pupal diapause, the hemolin concentration increased 18-fold. It was shown to specifically interact with the lipid A part of LPS. An X-ray structure was also determined, and it revealed immunoglobulin domains 2 and 3 as a phosphate-binding site, supporting the idea that the specificity of the bacterial interaction occurs through the diphosphate lipid A (Su *et al.*, 1998). The cellular defense effects of hemolin are reflected by its capacity to both inhibit hemocyte aggregation and enhance phagocytosis *in vitro*. Based on these findings, hemolin is considered a pattern-recognition molecule in the insect immune response. Several hemolins from different insects, including *M. sexta*, have also been purified and show similar bacterial binding specificities and

biological functions to those of giant silkworm hemolin (Yu and Kanost, 2002). Another LPS recognition protein (LRP), a novel 40-kDa protein, was purified to homogeneity from the large beetle *Holotrichia diomphalia*. LRP exhibited agglutinating activity on *Escherichia coli*, but not on *Staphylococcus aureus* or *Candida albicans*. This *E. coli*-agglutinating activity was preferentially inhibited by rough-type LPS with a complete oligosaccharide core. Interestingly, LRP consists of six repeats of an epidermal growth factor (EGF)-like domain. Furthermore, *E. coli* coated with purified LRP was cleared more rapidly in the *H. larvae* than naked *E. coli*, indicating that the protein participates in the clearance of *E. coli in vivo*. Three amino-terminal EGF-like domains of LRP, but not the three carboxyl epidermal growth factor-like domains, are involved in the LPS-binding activity, suggesting that LRP functions as a pattern-recognition protein for LPS and plays a role as an innate immune protein (Ju *et al.*, 2006).

14.4. Humoral Innate Immune Responses

14.4.1. Biochemical Properties of Insect Antimicrobial Peptides

Over the past two decades, many AMPs have been isolated from insects and plants (Broekaert *et al.*, 1995; Bulet *et al.*, 1999; Imler and Bulet, 2005). Most of these insect AMPs are classified into two groups: inducible AMPs that are secreted into the hemolymph when bacteria or fungi are injected into insects, and constitutive AMPs that exist in the naïve hemolymph. More than 250 insect AMPs have been isolated since the discovery of cecropin by the Boman group. In spite of great differences in size, amino acid composition, and structure, insect AMPs can be grouped into three categories. The largest AMPs belong to the first category: peptides with intramolecular disulfide bonds forming an α -helical- β -sheet, a hairpin-like β -sheet, or mixed structures. The second group comprises linear peptides forming amphipathic α -helices. The last category contains peptides with Pro- and/or Gly-rich residues.

Because the molecular activation and regulation mechanisms of insect AMP production are mainly studied in the *Drosophila* system, the biochemical characteristics of *Drosophila* AMPs will be summarized as a way to easily explain AMP induction signaling pathways, such as the Toll and IMD pathways (Lemaitre and Hoffmann, 2007). Many insect AMPs similar to those of *Drosophila* have been identified from a wide variety of different insects, and the relationships between their structures and activities are reviewed in other excellent papers (Brown and Hancock, 2006). The technical advances of analytical biochemistry and mass spectrometry enabled the purification of eight different AMPs using *Drosophila* hemolymph. These AMPs are grouped into three families based

on their antimicrobial activities. Specifically, defensin showed antibacterial activity against Gram-positive bacteria. Cecropin, drosocin, attacins, dipterin, and MPAC (matured pro-domain of attacin C) have antibacterial activity against Gram-negative bacteria. Drosomycin and metchnikowin are known to have antifungal activity. These peptides are inducible AMPs that are synthesized by the fat body in response to bacterial or fungal infections and then secreted into the hemolymph. The biochemical characteristics and functions of the eight *Drosophila* AMPs can be summarized as follows.

Defensin contains a 92-residue precursor containing an N-terminal signal peptide (20 residues) followed by a pro-domain (32 residues) and the mature defensin (40 residues). It consists of an α -helical domain linked to antiparallel β -strands with three internal disulfide linkages: Cys₁–Cys₄, Cys₂–Cys₅, and Cys₃–Cys₆. This disulfide bridge pattern is also observed in plant defensins but not in mammalian defensins, suggesting a difference in tertiary structures between invertebrate and vertebrate defensins. This AMP showed high homology with many invertebrate defensin-like AMPs, such as *Sarcophaga* sapecin A and *Tenebrio* tenecin 1. These insect defensins showed antibacterial activity against many Gram-positive bacteria, a limited number of Gram-negative bacteria, and some filamentous fungi. Insect defensins are reported to disrupt the permeability of the cytoplasmic membrane of bacteria, leading to the leakage of cytoplasmic potassium and partial depolarization of the inner membrane (Cociancich *et al.*, 1993). The antibacterial activity decreased in the presence of high salt concentration, as shown in the vertebrate and plant defensins.

Cecropins are synthesized as 63-residue precursors, with a putative signal peptide of 23 residues followed by mature cecropin (40 residues), and ending in a glycine residue that is amidated. Cecropin-like AMP is the first purified animal-inducible AMP generated in response to an experimental infection in diapausing pupae of the *H. cecropia* moth (Lepidoptera) (Steiner *et al.*, 1981). The three *Drosophila* cecropin genes (cecropin A–C) are also the first genes to be cloned. Synthetic *Drosophila* cecropin is highly efficacious on many Gram-negative bacteria at concentrations below 10 μ M, while most of the Gram-positive strains tested remained insensitive even at higher concentrations. It has been speculated that helix-forming cecropins induce the disintegration of bacterial membrane structures and lysis of bacteria.

Drosocin, which shows antibacterial activity against Gram-negative bacteria, is synthesized as a 64-residue precursor corresponding to a signal peptide (21 residues) followed by the mature drosocin (19 residues), and ending in a prodomain of 24 residues. This peptide has two biochemical characteristics: an *o*-glycosylated Thr residue, and three repeats of a Pro–Arg–Pro motif in the mature sequence. Apidaecins purified from honeybee *Apis*

mellifera are homologs of drosocin-like AMP (Casteels *et al.*, 1989). Unlike other α -helical- β -sheet- or hairpin-like β -sheet-forming AMPs, which work in a matter of minutes, drosocin takes several hours to kill bacteria. Interestingly, synthetic drosocin created with only D-type amino acid residues was totally inactive against Gram-negative bacteria, suggesting that drosocin has stereoselective bactericidal effects and is not only a pore-forming AMP.

Diptericin, which also displays antibacterial activity against Gram-negative bacteria, is synthesized as a 106-residue precursor containing signal peptide (23 residues) followed by the mature dipterin (83 residues). Similar peptides have been identified in other dipteran species, such as the blow fly *Prototophormia terraenovae*, the flesh fly *Sarcophaga peregrina* (Natori *et al.*, 1999), and the tsetse fly *Glossina morsitans* (Bulet *et al.*, 1999). The presence of *o*-glycosylation on the Thr residue within the N-terminal Pro-rich domain of this peptide was confirmed by biochemical methods. This *o*-glycosylation modification was also observed in drosocin and MPAC (see below). When full-size synthetic unglycosylated and *o*-glycosylated 82 mer *Prototophormia* dipterins were prepared by solid-phase synthesis (Cudic *et al.*, 1999) or expressed in an *E. coli* system (Winans *et al.*, 1999) and tested for antibacterial activity, the full-size synthetic *Prototophormia* dipterin showed antibacterial activity against only a limited number of Gram-negative bacteria. These bacteria were killed by increasing the permeability of the outer and inner membranes of *E. coli*.

Attacin homologs, at more than 190 amino acid residues in length, are the largest AMPs. These Cys-free attacin-like AMPs have no particular post-translational modification, and were initially reported in lepidopteran species with antibacterial activity against Gram-negative bacteria (Engström *et al.*, 1984). *Hyalophora* attacin has been shown to interfere with transcription of the *omp* gene of *E. coli*. The *omp* gene is involved in the synthesis of porines, which form protein channels in bacterial membranes (Carlsson *et al.*, 1991). MPAC (mature prodomain of attacin C) is synthesized as a 241-residue precursor containing a signal peptide (21 residues) followed by MPAC (23 residues). When a dibasic cleavage site of attacin C is cleaved, mature attacin C containing amidated Gly residue at the C-terminal is generated. MPAC is a Cys-free, Pro-rich 23 residue peptide containing N-terminus pyroglutamic acid and an *o*-glycosylation motif on the Thr residue similar to the Pro-rich drosocin. Amino acid comparisons showed that MPAC has high homology (37%) with the prodomains of dipteran dipterin, sarcotoxin IIA, and lepidopteran dipterins. Because MAPC showed high similarity to drosocin and Pro-rich AMPs from other insects, functional studies of MPAC were performed *in vitro* using synthetic non-glycosylated and glycosylated forms against several bacteria. Unexpectedly, none of the

strains appeared to be sensitive to MPAC, while a significant synergy was observed between MPAC and cecropin (Bulet *et al.*, 2003).

Drosomycin, which shows antifungal activity, is synthesized as a 70-residue precursor polypeptide containing a signal (26 residues) followed by the mature drosomycin (44 residues). Compared to insect defensin, which contains three disulfide linkages, drosomycin contains an additional disulfide linkage; the bonds are formed in the pattern Cys₁–Cys₈, Cys₂–Cys₅, Cys₃–Cys₆, Cys₄–Cys₇, suggesting the presence of an additional β -strand at the N-terminus of the molecule. Even though the six amino acid sequences of the *Drosophila* drosomycins do not show any similarities to those of the insect defensins, insect drosomycins are very similar to the plant defensins and r-thionins, two classes of defense molecules in plants (Padovan *et al.*, 2010). Drosomycins are potent antifungal peptides that inhibit the growth of filamentous fungi, which are pathogenic to humans and plants. Unlike insect defensins, drosomycins retain their biological activities at a high salt concentration.

Metchnikowin, another Cys-free and Pro-rich *Drosophila* antifungal peptide, is synthesized as a 52-residue precursor peptide containing a signal peptide (26 residues) followed by the mature metchnikowin (26 residues). The primary sequence of this AMP shows similarity to the C-terminal domains of dosocin, abaecin, and the lebo-cins, which are Pro-rich AMPs that have been purified from the fruit fly *D. melanogaster*, the bumblebee *Bombus pascuorum*, and the silkworm *B. mori*, respectively (Chowdhury *et al.*, 1995; Rees *et al.*, 1997). Recent studies have demonstrated that synthetic 26 amino acid metchnikowin caused strong growth inhibition of the pathogenic fungus *Fusarium graminearum* *in vitro*. Transgenic barley expressing the metchnikowin gene in its 52-aa form also displayed enhanced resistance to plant fungi, suggesting that antifungal peptides from insects can be a valuable source of crop plant improvements, and that insect peptides can be used as selective compounds against specific plant diseases (Rahnamaeian *et al.*, 2009). Finally, two Gly- and His-rich antifungal peptides named *Sarcophaga* antifungal protein (AFP) and holotricin-3 are purified from the hemolymph of the flesh fly *S. peregrina* (Iijima *et al.*, 1993) and the larvae of the coleopteran beetle, *H. diomphalia* (Lee *et al.*, 1995). Both peptides showed fungicidal activity against human *C. albicans*. However, the exact mode of action of these novel antifungal peptides is not yet clear.

14.4.2. Toll and IMD Signaling Pathways after Bacterial or Fungal Infection

14.4.2.1. Activation and regulation of *Drosophila* Toll and IMD signaling pathways Classical deletion-mapping studies on the cecropin A1 and dipterin genes began in 1990, to answer the question of how insect AMP

proteins are strongly induced or upregulated by microbial infection. The unique DNA sequences (GGGGATTYYT) identified in the cecropin A1 and dipterin promoter regions are specifically recognized by the Relish family of transcription factors, which belong to the NF- κ B protein family. This discovery led to the detection of this DNA motif in all other AMP genes (Engström *et al.*, 1993). Based on these *Drosophila* genome sequences, three different Relish family genes, Dorsal, Dif (Dorsal-related immunity factor), and Relish, were targeted for mutagenesis screening (Hetru and Hoffmann, 2009). Dif was shown to be essential for the induction of several *Drosophila* genes when flies were challenged with Gram-positive bacteria and fungi. Namely, *Dif* mutant flies strongly reduced the expression of drosomycin and defensin genes after microbial infection. The *Relish* gene was also induced in infected flies. The Relish protein contains two domains, an N-terminal Rel domain and a C-terminal I κ B-like ankyrin repeat domain. Upon infection, Relish was rapidly processed by the caspase Dredd molecule into two peptides: the Relish homology domain, which is translocated to the nucleus, and the ankyrin repeat domain, which remains cytosolic. This processing is essential for the expression of *Drosophila* AMPs. Dorsal-mutant larvae and flies exhibited a normal response to bacterial infection challenges. These basic studies provided important clues regarding the key roles the Relish family transcription factors play in the regulation of insect AMP genes via the Toll and IMD pathways.

The Toll pathway, named for the Toll transmembrane-associated receptor, was first genetically characterized for its role in the establishment of dorso-ventral polarity during *Drosophila* embryo development (Belvin and Anderson, 1996). The breakthrough genetic evidence of this pathway in insect immunology was reported between 2001 and 2003 by the group of Jules Hoffmann, which screened flies with mutant *Drosophila* PGRP-SA encoded by the gene *semmelweis*, and β -1,3-glucan recognition protein (β GRP)/Gram-negative binding protein-1 (GNBP1) encoded by the gene *osiris* (Michel *et al.*, 2001; Gobert *et al.*, 2003). Unexpectedly, loss-of-function mutations in either the PGRP-SA or the GNBP1 gene showed very similar phenotypes of compromised survival to Gram-positive bacterial infection, indicating that these two hemolymph proteins cooperate to sense Gram-positive bacteria and are essential for the activation of proteolytic enzymes(s) that cleave Spätzle, a Toll-receptor ligand. Conversely, when these two genes were overexpressed together, flies induced the activation of the Toll pathway even in the absence of a bacterial challenge (Gobert *et al.*, 2003). Hoffman's group also demonstrated that activation of Toll by fungal infection is independent of the *semmelweis* and *osiris* genes. A further genetic screen has identified the *persephone* gene, which encodes a

hemolymph trypsin-like serine protease that mediates the fungal-dependent cleavage of Spätzle and the activation of Toll. Overexpression of the *persephone* gene is sufficient to lead to Spätzle-dependent induction of the Toll pathway in the absence of an immune challenge (Ligoxygakis *et al.*, 2002a). Lately, Ferrandon and his colleagues have studied flies with mutant GGBP3, a protein encoded by the gene *hades*. They found that GGBP3 is a pattern-recognition receptor that is required for the detection of β -1,3-glucan, a fungal cell wall component. They also found a parallel pathway that acts jointly with GGBP3 (Gottar *et al.*, 2006). Specifically, when *Drosophila* persephone protease is proteolytically matured by the secreted fungal virulence protease PR1, it activates the Toll pathway. Thus, the detection of fungal infections in *Drosophila* relies both on the recognition of β -1,3-glucan, an invariant microbial cell wall component, and on the effects of virulence factors such as PR1 protease (Figure 2).

As shown in Figure 2, the *Drosophila* Toll signaling pathway is divided into three steps: (1) extracellular recognition of invading Gram-positive bacteria or fungi by the PGRP-SA/GGBP1 complex or GGBP3, respectively, and signaling amplification step by several serine proteases, leading to the cleavage of pro-Spätzle; (2) activation via binding between a Toll ligand, processed Spätzle and a Toll receptor; and (3) intracellular activation and the expression of effector molecules. Specifically, upon recognition of invading Gram-positive bacteria or fungi, recognition signals are amplified by the serine protease proteolytic cascade, as in mammalian blood coagulation or complement activation cascades (Krem and Di Cera, 2002). It has been suggested that the activation of pro-Spätzle is achieved by a set of serine proteases distinct from those involved in Toll activation during embryonic development. Recently, Lemaitre and colleagues identified modular serine protease (ModSP), the most upstream serine protease of *Drosophila* Toll cascade (Buchon *et al.*, 2009). They demonstrated that *Drosophila* ModSP integrates signals originating from the GGBP3 and PGRP-SA/GGBP1 complex, and connects them to the downstream serine proteases. The terminal serine protease that processes Spätzle has been identified, and named *Drosophila* Spätzle-processing enzyme (SPE) (Jang *et al.*, 2006). Even though several *Drosophila* clip-domain-containing serine proteases, such as Grass and Spirit, which are suggested to act between ModSP and SPE, have been identified, the biochemical functional studies of these serine proteases are not yet complete (Kambris *et al.*, 2006).

Upon binding of the cleaved Spätzle to the Toll receptor, the production of AMPs is induced from the fat body (Lemaitre and Hoffmann, 2007; Weber *et al.*, 2003). The Spätzle–Toll complex recruits a set of downstream molecules. First, Toll/IL-1 Receptor (TIR) and/or the death-domain-containing adaptor molecules dMyD88

and Tube lead to the activation of the kinase Pelle. Pelle induces the degradation of Cactus, an inhibitor that maintains the cytoplasmic localization of Dif and Dorsal, two transactivators of the NF- κ B family. The translocation of these transcription factors induces the expression of several immune genes, including AMP genes, through binding to the κ B DNA motifs of their promoter.

The IMD pathway is named after the first *Drosophila* mutant flies, called *immune deficiency* (IMD), which are susceptible to Gram-negative bacteria infection, but are more resistant to fungi and Gram-positive bacteria infection. The *imd* gene encodes a death-domain-containing protein similar to that of the receptor interacting protein (RIP) of the mammalian tumor necrosis factor receptor (TNFR) pathway (Georgel *et al.*, 2001). In 2002, three groups simultaneously reported the identity of the upstream receptor molecule of the *Drosophila* IMD pathway: PGRP-LC, a putative transmembrane protein that is required for the activation of the Gram-negative-mediated IMD pathway (Choe *et al.*, 2002; Gottar *et al.*, 2002; Rämét *et al.*, 2002). Compared to the IMD null mutant, loss-of-function mutants of PGRP-LC showed less severe phenotypes, suggesting that PGRP-LC is only one of several receptors that sense Gram-negative bacteria. Kurata and colleagues reported that *Drosophila* PGRP-LE, another member of the PGRP family, activates the IMD pathway when this gene is overexpressed (Takehana *et al.*, 2002). Taken together, these studies showed that PGRP-LC and PGRP-LE function as receptor molecules for the activation of the Gram-negative bacteria-mediated IMD pathway (Figure 2).

The intracellular signaling mechanism of the IMD pathway, mostly determined by genetic studies, is summarized as follows (Lemaitre and Hoffmann, 2007) (Figure 2): upon Gram-negative bacterial infection, PGRP-LC recruits the IMD protein, which interacts with the adaptor molecule dFADD via death-domain interaction. The dFADD then recruits the Dredd caspase, which is proposed to associate with Relish. After cleavage of the Relish protein, the Relish transactivator domain translocates to the nucleus, while the inhibitory domain of Relish remains in the cytoplasm. Relish that was phosphorylated by the inhibitory κ B-kinase signaling complex is proposed to be activated by Tak1 and its adaptor TAB2 in the IMD pathway. The molecular mechanisms behind the dimerization of PGRP-LC and the activation of the downstream signal pathway by the ligand–receptor complex have not yet been determined.

The serpins belong to a superfamily of serine protease inhibitors that act as suicide substrates by binding covalently to their target proteases. Serpins are known to regulate various physiological processes and defense reactions in mammals and invertebrates (Reichhart, 2005; Silverman *et al.*, 2010; Whisstock *et al.*, 2010). To date, four *Drosophila* serpins related to innate immunity

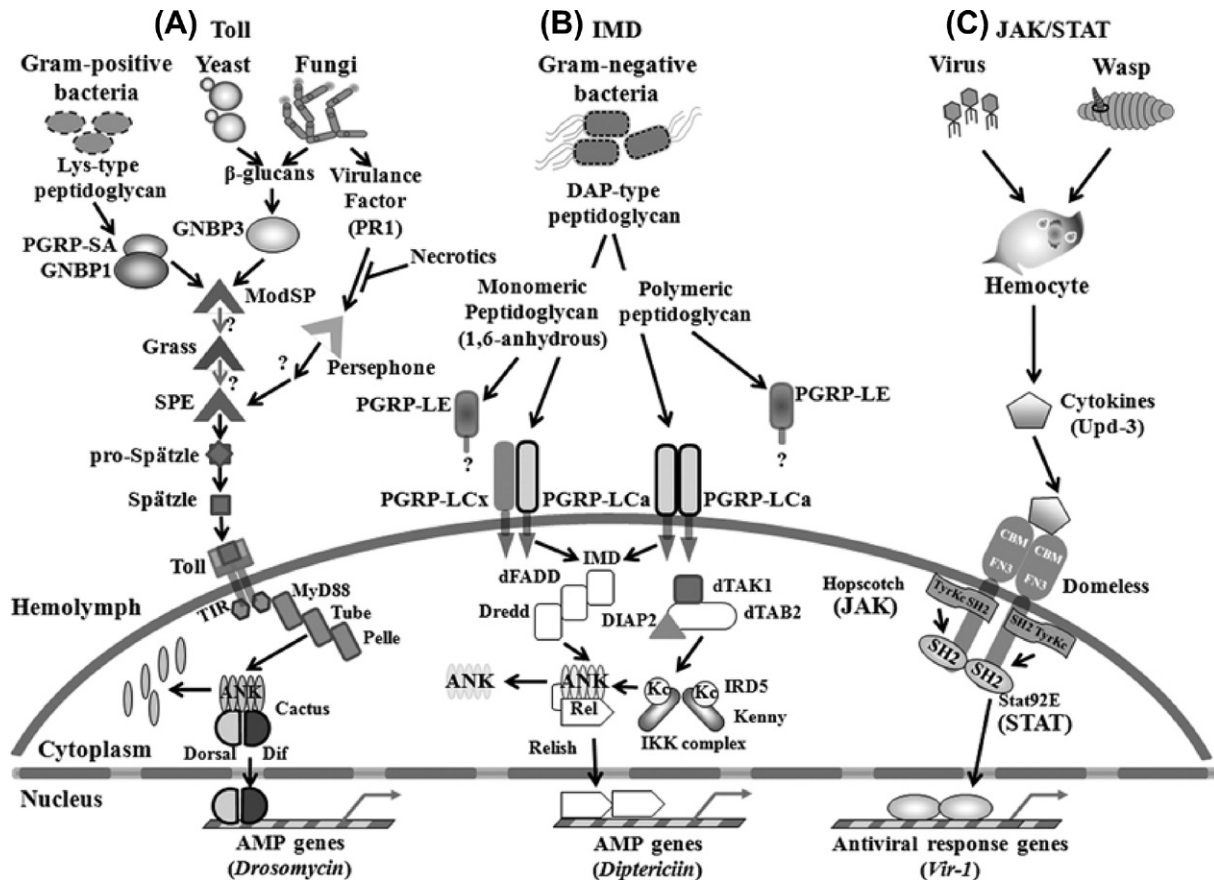


Figure 2 Three major *Drosophila* signaling pathways that regulate systemic immune responses against bacterial, fungal and viral infection. (A) The Toll pathway is activated by the Lys-type peptidoglycan of Gram-positive bacteria and β -1,3-glucan of yeast and some fungi through two different pattern recognition proteins, such as PGRP-SA/GNBP1 and GNBPs, respectively. These recognition signals initiate the activation of the proteolytic serine protease cascade via ModSP, Grass, and Spätzle-processing enzyme (SPE), which are clip-domains containing serine protease, leading to cleavage of pro-Spätzle to the processed Spätzle by activated SPE. Upon binding processed Spätzle to Toll, dimerized Toll recruits dMyD88, Tube, and Pelle, resulting in the phosphorylation and proteosomal degradation of Cactus. Cactus degradation induces the translocation of Rel transcription factors, Dif and Dorsal, to the nucleus. These factors bind to NF- κ B-response elements and induce activation of transcription of AMP genes, such as Drosomycin. The virulence factor of fungi, such as PR1 protease, is also suggested to activate pro-SPE to activated SPE, leading to the cleavage of pro-Spätzle to the processed Spätzle. (B) The polymeric and monomeric DAP-type peptidoglycans of Gram-negative bacteria are recognized by PGRP-LE and PGRP-LCs. These recognition signals are transferred to the IMD, which is localized in cytoplasm. Upon binding of monomeric DAP-type peptidoglycan to PGRP-LCx/LCa heterodimer or polymeric DAP-type peptidoglycan to PGRP-LCx homodimer, IMD is recruited by the intracellular domain of PGRP-LCs. IMD then recruits dFADD and caspase Dredd, which cleave the phosphorylated Relish, leading to translocation of Rel domain to nucleus, where the Rel domain binds to NF- κ B response elements and activates the transcription of AMP genes, such as Diptericin. The phosphorylation of Relish is suggested to be mediated by an inhibitory κ B (IKK) complex (containing IRD5 and Kenny). dTAK1 activation is induced by adaptor dTAB2 and DIAP2. (C) Upon binding of cytokine Upd3, which is secreted from hemocytes into hemolymph by viral and wasp-parasitoid infection, to the dimerized Domeless receptors, JAK (Hopsotch) is activated. The JAK recruits the STATs (Stat92E), which are phosphorylated and dimerized, leading to translocation to the nucleus to activate transcription of target genes, such as *Vir-1*.

(SPN43Ac, SPN27A, SPN77Ba, and SPN28D) have been analyzed in detail by genetic approaches. *SPN43Ac* mutant flies accumulated cleaved Spätzle, resulting in constitutive activation of the Toll pathway and the expression of AMPs (Levashina *et al.*, 1999). *SPN27A* and *SPN28D* are known to regulate the Toll pathway during early development (Hashimoto *et al.*, 2003; Ligoxygakis *et al.*, 2003; Scherfer *et al.*, 2008), and are also involved in the melanin biosynthesis reaction (De Gregorio *et al.*,

2002; Ligoxygakis *et al.*, 2002b). However, the molecular identities of the serpin target serine proteases and the biochemical regulatory mechanisms of these serpins have not been clearly demonstrated, leading to a lack of molecular understanding of the roles of serpins in the Toll signaling cascade.

The regulatory mechanisms of the *Drosophila* IMD pathway have also been studied; four negative regulators of the IMD pathway have been identified and characterized.

PGRP-LF, a membrane-bound non-catalytic PGRP containing two PGRP domains, was demonstrated to be a key negative regulator of the PGRP-LC-mediated IMD signaling pathway (Maillet *et al.*, 2008). However, the inhibition of the IMD pathway by PGRP-LF was induced even in the absence of infection, allowing the prevention of aberrant activation of the IMD pathway by residual PGN fragments that are ingested from food or released by indigenous microbes. Pirk (poor IMD response upon knock-in), a protein interacting with PGRP-LC, is also known as Rutra (a new regulator of the IMD pathway); PIMS (PGRP-LC-interacting inhibitor of IMD signaling) is another negative regulator of the IMD pathway. The biological functions of these proteins are involved in the precise control of IMD pathway induction, but the exact biological functions are still not clear (Aggarwal *et al.*, 2008; Kleino *et al.*, 2008; Lhocine *et al.*, 2008). Furthermore, catalytic PGRPs such as PGRP-SC1 and PGRP-LB, which show amidase activity against PGN, were reported as another family of negative regulators of the IMD pathway. These PGRPs function as scavengers by cleaving the stem-peptide of PGN, thereby eliminating the immune-stimulating activity of PGN and leading to shutdown of the PGRP-LC-mediated IMD signaling pathway (Bischoff *et al.*, 2006).

Although we have observed how *Drosophila* Toll and IMD pathways are activated through microbe-recognition proteins and their extracellular and intracellular adaptor molecules after recognition of Gram-positive, Gram-negative bacteria or fungi, it is necessary to answer the question of which PAMP molecules of these microbes can activate these signaling pathways. In 2003, Lemaitre and his colleagues first reported the determination of ligand molecules of the *Drosophila* Toll and IMD pathways, demonstrating that DAP-type PGN of Gram-negative bacteria and certain *bacilli* species function as the most potent activators of the IMD pathway, while the Toll pathway is predominantly activated by Lys-type PGN of Gram-positive bacteria (Leulier *et al.*, 2003). These results clearly demonstrated that the discrimination between Gram-positive and Gram-negative bacteria in *Drosophila* relies on the recognition of specific forms of PGN but not other bacterial cell wall components, such as LPS, wall teichoic acid, lipoteichoic acid, and lipoprotein.

14.4.2.2. Activation and regulation of *Tenebrio* Toll signaling pathway As shown above, *Drosophila* genetics are very powerful tools for characterizing and ordering the components in the *Drosophila* Toll and IMD signaling pathways. However, this system is still limited in terms of determining the biochemical mechanisms involved in regulating the proteolytic Toll cascade. Since *Drosophila* has several alternative routes to the Toll pathway, used at various developmental stages and infection protocols, it is difficult to determine the clear activation mechanism

of the Toll signaling cascade. For instance, *Drosophila* persephone is another serine protease linked to the Toll pathway and antifungal immunity, yet the biological functions of this molecule have only been partially characterized by *Drosophila* genetic studies. The proper identification of downstream factor(s) of persephone still awaits further investigation. To provide compelling biochemical data on how the Lys-type PGN and β -1,3-glucan recognition signals can be sequentially transferred to Spätzle during the Toll signaling pathway, it is necessary to use a larger insect that enables us to collect larger amounts of hemolymph. The coleopteran larvae *T. molitor* was used for intensive biochemical studies, resulting in the purification of nine proteins, which are involved in the activation of the *Tenebrio* Toll signaling pathway (Park *et al.*, 2007; Kim *et al.*, 2008; Roh *et al.*, 2009) (Figure 3). The nine molecules include three pattern-recognition proteins (*Tenebrio* PGRP-SA, GNB1-1, GNB1-3), three serine protease zymogens (*Tenebrio* modular serine protease (MSP); Spätzle processing enzyme (SPE), and SPE-activating enzyme (SAE)), and recombinant pro-Spätzle and recombinant Toll-ecto domain-containing proteins, which were purified to homogeneity. The activation mechanism of the *Tenebrio* Toll signaling pathway was then studied biochemically using *in vitro* reconstitution experiments. We proposed that the *Tenebrio* PGRP-SA/GNB1-1/MSP/SAE/SPE/Spätzle cascade is an essential unit that triggers the Lys-type PGN recognition signaling pathway in response to Gram-positive bacterial infection in the *Tenebrio* system (Kim *et al.*, 2008).

The β -1,3-glucan recognition signal is also transferred via the sequential activation of the three *Tenebrio* serine proteases, MSP, SAE, and SPE, which leads to the processing of pro-Spätzle to its mature form Spätzle, demonstrating that a three-step proteolytic cascade is essential for Toll pathway activation by fungal β -1,3-glucan in *Tenebrio* larvae. This cascade is shared with Lys-type PGN-induced Toll pathway activation (Roh *et al.*, 2009). Furthermore, we demonstrated that β -1,3-glucan and Lys-type PGN activate the Toll signaling cascade using the same three-step proteolytic cascade that results in the production of two *Tenebrio* AMPs, tenecin 1 and tenecin 2. The amino acid sequence of tenecin 1 and its disulfide bond arrangement were found to be very similar to *Drosophila* defensin, while tenecin 2 showed high sequence identity (65% and 36%) with coleoptericin and holotricin-2, respectively. Holotricin-2, previously identified by our group, is also an inducible antibacterial peptide purified from coleopteran *H. diomphalia* larvae (Lee *et al.*, 1994). The molecular mechanisms behind the recognition of Gram-positive bacteria or fungi by *Tenebrio* larvae *in vivo*, the activation of the Toll pathway, and the type of AMPs induced after the activation of the bacteria- or fungi-mediated Toll signaling cascade are clearly determined. Finally, the upstream

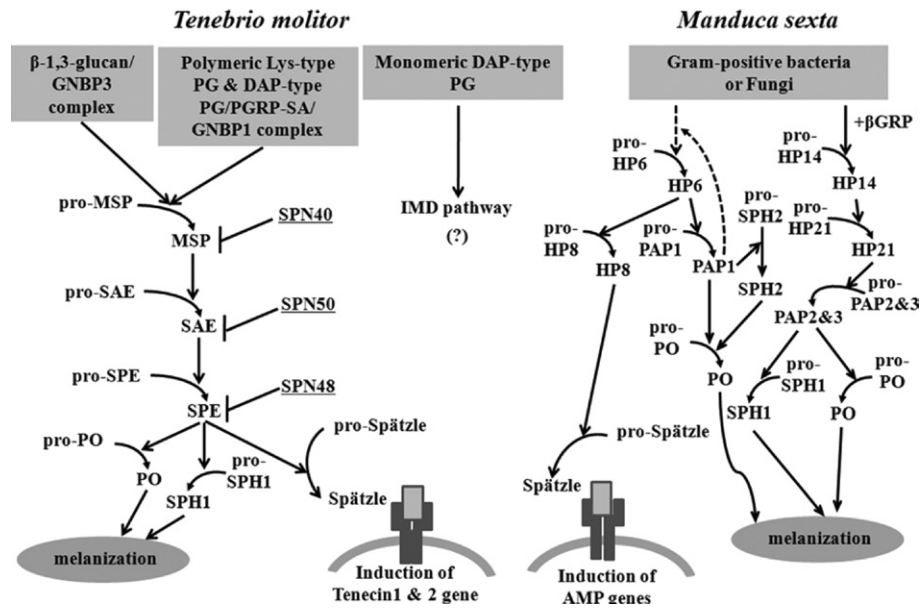


Figure 3 Comparison of *Tenebrio* and *Manduca* extracellular proteolytic Toll signaling pathways. (A) The *Tenebrio* Toll signaling pathway shares a three-step proteolytic cascade, which consists of three serine proteases, such as modular serine protease (MSP), Spätzle-processing enzyme (SPE), and SPE activating enzyme (SAE). Lys-type peptidoglycan and β -1,3-glucan are recognized by the *Tenebrio* PGRP-SA/GNBP1 complex and GNBP3, as in the *Drosophila* system. The recognition signals induce three serine proteases downstream. The activated SPE leads to the cleavage of pro-Spätzle to processed Spätzle, leading to the activation of the Toll cascade and, subsequently, production of the *Tenebrio* AMPs, tenecin 1 and 2. Three serpins (SPN 40, SPN 55, and SPN 45) make specific three serpin-serine protease complexes, and inhibit the processing of pro-Spätzle and phenoloxidase-mediated melanin synthesis. Polymeric DAP-type peptidoglycan of Gram-negative bacteria in *Tenebrio* system also uses the same cascade as that of the polymeric Lys-type peptidoglycan-mediated Toll signaling pathway. (B) The production of AMP and melanin synthesis in *Manduca* system is separated by two branches. *Manduca* AMP production begins with hemolymph protease 6 (HP6). The activated HP6 activates proHP8 to the active form of HP8, which can cleave pro-Spätzle to Spätzle, resulting in the induction of *Manduca* AMPs. The Michael Kanost group of Kansas State University proposed that activated HP6 can also activate prophenoloxidase activating proteinases-1 (PAP-1), which can cleave *Manduca* prophenoloxidase to phenoloxidase, leading to melanin synthesis in the presence of serine protease homolog 2. Also, *Manduca* prophenoloxidase is activated by a β -1,3-glucan recognition protein (β GRP)-mediated three-step proteolytic cascade (HP14/HP21/PAP2&3), leading to melanin synthesis in the presence of SPH1.

pathogen recognition features of the *Tenebrio* Toll cascade are reminiscent of the complement activation by the lectin pathway in mammals in which the recognition of carbohydrates by mannose-binding lectin (MBL) leads to the auto-activation of MBL-associated serine proteases (MASPs) (Matsushita and Fujita, 1992). The domain organization of MASPs is similar to those of insect MSPs.

Recently, the recognition mechanism of DAP-type PGN in the *Tenebrio* system has also been proposed (Yu *et al.*, 2010), although the *Drosophila* IMD pathway responds to polymeric and monomeric DAP-type PGNs of Gram-negative bacteria and certain Gram-positive *Bacillus* species through PGRP-LC or PGRP-LE receptors. Unexpectedly, polymeric (but not monomeric) DAP-type PGN formed a complex with *Tenebrio* PGRP-SA, and this complex activated the three-step proteolytic cascade to produce processed Spätzle, leading to the induction of tenecin 1 in *Tenebrio* larvae, as in the Lys-type PGN-mediated Toll pathway (Figure 3). In addition, both polymeric and monomeric DAP-type PGNs induced the expression

of *Tenebrio* PGRP-SC2, which is a DAP-type PGN-selective *N*-acetylmuramyl-L-alanine amidase that functions as a DAP-type PGN scavenger. PGRP-SC2 appears to function as a negative regulator of DAP-type PGN signaling by cleaving DAP-type PGN, rendering it incapable of inducing AMPs in *Tenebrio* larvae. These results demonstrated that the molecular recognition mechanism for polymeric DAP-type PGN differs between *Tenebrio* larvae and *Drosophila* adults, providing biochemical evidence of biological diversity in the innate immune responses of insects.

Because three serine proteases that are directly involved in the activation of the *Tenebrio* Toll cascade were identified, *Tenebrio* larvae were assumed to be a useful system for identifying and characterizing novel target serpins that directly regulate the Toll proteolytic cascade. As described above, because the molecular identities of the *Drosophila* target serine proteases of the four *Drosophila* serpins and the biochemical regulatory mechanisms of these serpins were not clearly demonstrated, we tried to understand the molecular regulation mechanism biochemically

using the *Tenebrio* system. Three novel serpins (SPN40, SPN55, and SPN48) from the hemolymph of *T. molitor* larvae were purified (Jiang *et al.*, 2009). These serpins made specific serpin–serine protease pairs with three Toll cascade-activating serine proteases (MSP, SAE, and SPE), and cooperatively blocked the Toll signaling cascade and β -1,3-glucan-mediated melanin biosynthesis. In addition, the levels of SPN40 and SPN55 were dramatically increased *in vivo* by the injection of the processed Spätzle into *Tenebrio* larvae. This increase in SPN40 and SPN55 levels indicates that these serpins function as inducible negative feedback inhibitors. In addition, SPN55 and SPN48 were cleaved at Tyr and Glu residues in reactive center loops, respectively, despite being targeted by trypsin-like SAE and SPE serine proteases. These cleavage patterns are also highly similar to those of the unusual mammalian serpins involved in blood coagulation and blood pressure regulation, and they may contribute to highly specific and timely inactivation of detrimental serine proteases during innate immune responses. It had been thought that the *Drosophila* Toll cascade was regulated by the activity of a single “bottle-neck” protease inhibitor, but our data presented the first indication that each individual protease in a cascade may be regulated by a specific serpin (Figure 3).

14.4.2.3. *Manduca* Toll signaling pathway The Toll cascade of another large insect was also studied biochemically. The Kanost group characterized more than 20 clip-domain-containing serine proteases in the hemolymph of the tobacco hornworm, *M. sexta* (Jiang and Kanost, 2000). Recently, they reported the function of two *Manduca* serine proteases, hemolymph proteases 6 and 8 (HP6 and HP8; An *et al.*, 2009; see also Figure 3). HP6 and HP8 are each composed of an N-terminal clip domain and a C-terminal serine protease domain. HP6 was an apparent ortholog of *Drosophila* persephone, whereas HP8 was most similar to *Drosophila* and *Tenebrio* SPE, all of which activate the Toll pathway. Recombinant HP6 was found to activate prophenoloxidase-activating proteinase (proPAP1) *in vitro* and induce prophenoloxidase activation in plasma. HP6 was also determined to activate proHP8. Active HP6 or HP8 injected into larvae induced the expression of AMPs such as attacin, cecropin, and lysozyme. These results suggest that proHP6 becomes activated in response to microbial infection, and participates in two immune pathways: activation of PAP1, which leads to prophenoloxidase activation and melanin synthesis, and activation of HP8, which stimulates the *Manduca* Toll-like signaling pathway. However, the most upstream receptors of the Toll pathway, such as *Manduca* PGRP-SA and GNBPI, have not yet been characterized in the *Manduca* system.

The Kanost group has done pioneering work in the biochemical characterization of insect serpins. They first

described the *M. sexta* serpin-1, which has 12 different copies of exon 9 that undergo mutually exclusive alternative splicing to produce 12 putative protein isoforms. These isoforms differ in their carboxyl-terminal 39–46 residues, including the P1 residue, and inhibit serine proteases with different specificities (Kanost and Jiang, 1997). Recently, they also reported the biological function of the *Manduca* serpins: for example, serpin-1 isoforms can inhibit HP8, which activates pro-Spätzle, suggesting that serpin-1 isoforms may be involved in regulation of the *Manduca* Toll cascade (Ragan *et al.*, 2010). The proposed model of activation and regulation of the *Manduca* Toll cascades is shown in Figure 3.

14.4.3. Lectin Induction

Lectins are defined as a protein family capable of recognizing specific oligosaccharides, which were initially purified from various plant seeds. Similar proteins have also been isolated from a number of organs in a wide range of vertebrates and invertebrates (Barondes, 1984). Because the diverse biological roles of purified insect lectins cannot all be mentioned in this chapter, we will focus on the insect lectins that mainly participate in insect immunology. The pioneering work was performed by Natori and his colleagues using *S. peregrina* flesh fly larvae (Natori *et al.*, 1999). *Sarcophaga* lectins functioning as defense proteins are synthesized by the fat body and secreted into the hemolymph when the larval body wall is pricked with a hypodermic needle (Okada and Natori, 1983). This 260-aa lectin is a typical C-type lectin that requires calcium ions for agglutinating activity. C-type lectins, a superfamily of calcium-dependent carbohydrate-binding proteins, are known to function in pathogen recognition, cell–cell interactions, and innate immunity in mammals (Weis *et al.*, 1998). This lectin is rapidly induced when sheep red cells are injected, but is not present in the hemolymph of naïve larvae. Elegant biochemical studies performed by the Natori group demonstrated that this lectin has dual functions in defense and development, and provided evidence that the *Sarcophaga* lectin activates insect hemocytes in some way, resulting in the activation of hemocyte-mediated digestion of non-self foreign cells (Nakajima *et al.*, 1982; Komano *et al.*, 1983). They showed that this lectin was also needed for the differentiation of imaginal discs in the pupal stage. When antibodies against *Sarcophaga* lectin were added to the culture medium of *Sarcophaga* imaginal discs, differentiation was strongly inhibited; none of the discs reached the stage of terminal differentiation (Kawaguchi *et al.*, 1991). These results suggest that *Sarcophaga* lectin is not simply a defense molecule that is needed for the elimination of the invading non-self cells, but also a regulatory molecule in the development of imaginal discs.

Kanost and his colleagues also purified four soluble C-type lectins with two carbohydrate-recognition domains from the tobacco hornworm *M. sexta*, and named them immulectin-1 through -4 (Yu *et al.*, 1999). Expression of all four immulectins is upregulated in the fat body upon bacterial challenge. Immulectin-1 and -4 agglutinate Gram-negative and Gram-positive bacteria and yeast. However, immulectin-2 binds to a wide range of microbial cell wall components such as lipoteichoic acid, laminarin (branched β -1,3-glucan), mannose, and LPS. Furthermore, knockdown of the immulectin-2 gene at the level of both mRNA and protein by RNA interference (RNAi) markedly decreased the ability of *M. sexta* to defend bacterial infection when exposed to either species of the insect pathogen *Photobacterium*, suggesting that the *Manduca* lectin is not the only one that recognizes *Photobacterium*; the lectin-mediated insect immune system also plays an essential role in defending bacterial infection (Eleftherianos *et al.*, 2006). Similar tandem-domain C-type lectins are identified in other lepidopterans, and are involved in bacterial binding and hemocyte aggregation (Koizumi *et al.*, 1999). However, further studies are necessary to determine the structure of the ligand molecules and the biological functions of these C-type insect lectins during activation of insect innate immunity.

Insect galectin homologs, which have the ability to bind β -galactoside sugars, are identified from two insects, *D. melanogaster* (Pace *et al.*, 2002) and *A. gambiae* (Dimopoulos *et al.*, 1997). Fourteen galectins have been identified in mammals (Rabinovich *et al.*, 2002). However, the identification of precise biological functions for these mammalian galectins is difficult because of the redundancy in tissue expression and the complexity of recognition mechanisms in the target cells. A benefit of working with insect systems such as *Drosophila* and *Anopheles* is the ease of genetic manipulation and the rapid generation time. In addition, there are relatively small numbers of putative galectins in the *Drosophila* and *Anopheles* genomes; lower organisms such as insects are therefore useful in deciphering the precise biological functions of galectins. The Kafatos group demonstrated that a putative galectin homolog was upregulated in the salivary and gut of *A. gambiae* when mosquitoes were infected with malaria and bacteria. The *Anopheles* galectin was suggested to function as a pattern-recognition protein by binding saccharide ligands on the microbial cell wall surface to trigger a host innate immune response (Dimopoulos *et al.*, 2001). However, *Drosophila* galectin was expressed in naïve hemocytes, but not by the fat body or larval hemolymph (Pace *et al.*, 2002). Mammalian galectins were suggested to participate in the innate immune response by facilitating microbial recognition and/or lectin-mediated phagocytosis (Fradin *et al.*, 2000; Rahnamaeian *et al.*, 2009). The elucidation of Toll receptor-mediated immune response in insects has led to rapid progress in the understanding of innate immune

function in mammals; the determination of biological functions of insect galectins may similarly provide insight into the novel biological functions of mammalian galectins.

14.4.4. Melanin Synthesis

The melanization reaction is a major humoral immune response in insects and arthropods. Biochemical studies using large insects, which enable the collection of large amounts of hemolymph for biochemical studies, have defined the molecular basis of melanization activation and its regulation upon pathogenic microbe infection. These studies, along with molecular genetic analysis of melanization in the fruit fly and mosquito, have provided new insight into its biological role in fighting microbe infections (Cerenius *et al.*, 2008, 2010).

14.4.4.1. Melanin synthesis by bacterial and fungal infection Melanization of the insect cuticle was observed by Pasteur in 1870 while studying silkworm disease. In 1927, Metalnikov observed the hemolymph melanization of the wax moth *G. mellonella* and assumed that the formation of the dark pigment was the result of the action of a special enzyme on a chromogenic substrate found in the insect blood. In 1953, Ohnishi discovered the inactive zymogen form of phenoloxidase and its proteinaceous activator in *Drosophila* hemolymph (Ohnishi, 1953). Then, in 1955, Mason defined phenoloxidase as a copper-containing enzyme that catalyzes two reactions: oxygenation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones. These reactions are key steps in the synthesis of the black melanin pigment (Mason, 1955). In 1974, Pye reported that bacteria (*Pseudomonas aeruginosa*) and zymosan (the yeast cell wall components mainly composed from β -1,3-glucan and mannan) trigger the activation of prophenoloxidase in *G. mellonella* hemolymph (Pye, 1974). At the time, it was unknown whether bacteria or zymosan directly activated prophenoloxidase, or if a proteolytic activation cascade was involved. Subsequently, the Ashida group intensively studied the basic activation mechanisms of the prophenoloxidase cascade system using the silkworm *B. mori* (Ashida *et al.*, 1974).

The Ashida group demonstrated that *Bombyx* prophenoloxidase is activated when silkworm plasma is incubated with β -1,3-glucan from fungi or PGN from bacteria (Ashida *et al.*, 1983; Yoshida *et al.*, 1986), leading to the purification and cDNA cloning of the so-called insect PGRP and β -1,3-glucan recognition protein (β GRP) from silkworm hemolymph (Ochiai and Ashida, 2000). These pioneering works provided important biochemical evidence that the invertebrate prophenoloxidase activation system may be activated by elicitors derived from microbial cell walls such as PGN and β -1,3-glucan. The prophenoloxidase system, like the complementary

vertebrate system, is a proteolytic cascade containing several serine proteases and their inhibitors, and terminates with the prophenoloxidase zymogen. Microbial carbohydrates such as PGN or β -1,3-glucan first react with pattern-recognition proteins, such as PGRP or β GRP, which then induce activation of several serine proteases within the prophenoloxidase system.

In 1986, Collins *et al.* (1986) reported the important discovery that refractoriness to malaria parasites in the mosquito *A. gambiae* is correlated with melanization of the malaria ookinetes before they develop to the oocyst stage. This unique internal defense mechanism has received considerable attention, because this melanization reaction could be exploited to control and treat mosquito-borne diseases. Following this report, major research efforts have been focused on the identification of genes and gene products that are associated with the melanization response, with the intention of manipulating susceptible mosquito strains to be more resistant to malaria. The detailed results are mentioned below.

In 1995, the complete amino acid sequences of prophenoloxidase from three insect species (silkworm *B. mori*, tobacco hornworm *M. sexta*, and fruit fly *D. melanogaster*) were deduced from the respective cDNA sequences by three independent groups (Fujimoto *et al.*, 1995; Hall *et al.*, 1995; Kawabata *et al.*, 1995). Surprisingly, the deduced sequences clearly indicated that insect prophenoloxidase is a protein homolog to arthropod hemocyanin; the conserved histidine residues of the arthropod hemocyanins perfectly aligned with those in the insect prophenoloxidases and sequence homology ranged from 30% to 40%. A recent crystal structure of *M. sexta* prophenoloxidase shows that this unique insect oxidase makes a heterodimer consisting of two similar polypeptide subunit chains, prophenoloxidase-1 and -2. The enzyme active site of each subunit contains a typical type-3 dinuclear copper center; each copper ion coordinates with three structurally conserved histidine residues. The acidic glutamic acid residue located at the active site of prophenoloxidase-2 serves as a general base that deprotonates monophenolic substrates. This step is necessary for the *o*-hydroxylase activity of the generated phenoloxidase, and allows us to propose a model for localized prophenoloxidase activation in insects (Li *et al.*, 2009).

After the purification of the first prophenoloxidase-activating enzyme (PPAE) from the cuticle of silkworm (Dohke, 1973), the entire domain structure of insect PPAE, which is a clip-domain-containing serine protease, was determined in 1998 (Jiang *et al.*, 1998; Lee *et al.*, 1998). The clip-domain-containing serine protease-mediated proteolytic cascade was previously studied in the hemolymph coagulation cascade of the horseshoe crab *Tachypleus tridentatus* (Iwanaga *et al.*, 1992). Clip-domains have also been found in the *Drosophila* snake and easter precursor proteins, which are both indispensable

proteins for normal embryonic development (Chasan and Anderson, 1989). These serine proteases are synthesized and secreted into the hemolymph as inactive zymogens, which are then activated at a specific cleavage site by another upstream protease. The activation of most prophenoloxidases by PPAE induces the cleavage of a conserved Arg-Phe bond in prophenoloxidase, resulting in the removal of the N-terminal fragment consisting of about 50 amino acid residues (Fujimoto *et al.*, 1995; Kim *et al.*, 2002). The generated phenoloxidase was assumed to make a polymer with melanin synthesis activity. An additional serine protease homolog (SPH), a non-catalytic enzyme with an active Ser to Gly mutation within the N-terminal clip-domain, was required for the activation of prophenoloxidase and for phenoloxidase activity (Kwon *et al.*, 2000). Similar SPHs are identified in other insects, and are involved in the activation of the prophenoloxidase cascade (Yu *et al.*, 2003). The crystal structures and the functional roles of one clip-domain-containing PPAE and one SPH during the prophenoloxidase activation cascade were resolved (Piao *et al.*, 2005, 2007), demonstrating that insect SPH forms a homooligomer upon cleavage by the upstream protease, and that the clip-domain of SPH functions as a module for binding phenoloxidase through the central cleft, while the clip-domain of a serine protease plays an essential role in the rapid activation of its protease domain.

Recently, our lab, as well as the Kanost group, has intensively studied the molecular activation mechanisms of the insect prophenoloxidase cascade at the molecular level using biochemical methods with the meal worm *T. molitor* and with *M. sexta* larvae (Kan *et al.*, 2008; An *et al.*, 2009). As shown in Figure 3, we provided clear biochemical evidence of the *Tenebrio* melanin synthesis cascade: *Tenebrio* Spätzle processing enzyme (SPE) cleaves both the 79-kDa *Tenebrio* prophenoloxidase and the *Tenebrio* clip-domain SPH zymogen to an active melanization complex. This complex, consisting of the 76-kDa *Tenebrio*-activated phenoloxidase and an active form of *Tenebrio* clip-domain SPH, efficiently produces melanin pigment on the surface of bacteria, to strong bactericidal effect. Additionally, we found the phenoloxidase-induced melanization reaction to be tightly regulated by *Tenebrio* prophenoloxidase itself, which functions as a competitive inhibitor of melanization complex formation. Our results demonstrate that the *Tenebrio* Toll signaling pathway and the melanization cascade share a common serine protease cascade for the regulation of these two major humoral innate immune responses. In the *Manduca* melanin synthesis cascade, an initiation proteinase precursor, proHP14, is autoactivated in response to Gram-positive bacterial or fungal infection. HP14 activates proHP21; HP21 activates proPAP2 or proPAP3; PAP2 or PAP3 then cleaves prophenoloxidase to form active phenoloxidase in the presence of SPH1 and

SPH2. Activation of prophenoloxidase can also be catalyzed by PAP1 when the high molecular SPH complex is also present. PAP1 also activates proSPH2 directly, and can indirectly lead to proHP6 activation (**Figure 3**).

Melanization in insects is crucial for defense and development, but must be tightly controlled. Systemic hyperactivation of the prophenoloxidase system, excessive formation of quinones, and inappropriate excessive melanin synthesis are deleterious to the hosts, suggesting that the prophenoloxidase activation system and melanin formation should be tightly regulated by serpins or melanization-regulatory molecules or inhibitors. Three different serpins (*Drosophila* Spn27A, Spn28D, and Spn77Ba) that regulate *Drosophila* melanization have been identified (Silverman *et al.*, 2010). Spn27A was the first serpin identified to regulate activation of phenoloxidase and melanization in the hemolymph. A loss-of-function mutation in Spn27A resulted in uncontrolled melanization as well as in semi-lethality, while overexpression of Spn27A suppressed phenoloxidase activation induced by microorganisms (De Gregorio *et al.*, 2002; Ligoxygakis *et al.*, 2002b). The target protease of Spn27A in the *Drosophila* melanization cascade has not yet been determined, but in an *in vitro* experiment, recombinant Spn27A was able to make a protease–serpin complex with the PPAE from the beetle *H. diomphalia* (De Gregorio *et al.*, 2002). Another serpin, Spn28D, inhibited phenoloxidase activation at a different level than Spn27A (Scherfer *et al.*, 2008). Knockdown of Spn28D by RNAi led to constitutive melanization in various tissues. Spn28D may function to prevent premature activation of phenoloxidase at an early stage, and confine its general availability. The serpin Spn77Ba was discovered to be a regulator of melanization in the tracheal respiratory system of *Drosophila* (Tang *et al.*, 2008). Spn77Ba was expressed in the tracheal epithelium, and knockdown of Spn77Ba specifically in trachea led to constitutive tracheal melanization and associated lethality.

Several serpins have been purified from the large insects *M. sexta* and *T. molitor*, and characterized as negative regulators of the insect prophenoloxidase system (Jiang and Kanost, 1997; Jiang *et al.*, 2009). In the *Manduca* system, the Kanost group reported elegant results regarding serpin splicing: 12 different copies of *Manduca* serpin 1 undergo mutually exclusive alternative splicing to produce 12 putative protein isoforms, which differ in the carboxyl-terminal residues 39–46, including the P1 residue. These serpins inhibited *Manduca* serine proteases with different specificity (Ragan *et al.*, 2010). These serpins were characterized, and suggested as negative regulators of the prophenoloxidase and Toll signaling cascades (Kanost *et al.*, 2004). Three novel serpins (SPN40, SPN55, and SPN48) were purified from the hemolymph of *T. molitor* and characterized (Jiang *et al.*, 2009) (**Figure 3**). These *Tenebrio* serpins

made specific serpin–serine protease complexes with the three Toll signaling cascade-activating serine proteases MSP, SAE, and SPE, and cooperatively blocked the *Tenebrio* Toll-signaling cascade and β -1,3-glucan-mediated melanin biosynthesis. Inhibitors that are directly involved in the inhibition of the enzymatic activity of phenoloxidase are also identified as regulators of melanization. Phenoloxidase inhibitor (POI) was purified biochemically in the housefly *Musca domestica* (Daquinag *et al.*, 1995). POI homologs also have been identified in *M. sexta* (Lu and Jiang, 2007), and in the mosquito *A. gambiae* (Shi *et al.*, 2006). When *Anopheles* POI was knocked down using RNAi, melanin synthesis was suppressed. In *T. molitor*, a novel 43-kDa melanization-inhibiting protein (MIP) was found to specifically inhibit melanin synthesis (Zhao *et al.*, 2005). The biological function of MIP was proposed to negatively regulate phenoloxidase activation, and thus prevent possible tissue damage. No similar protein showing significant sequence homology to the *Tenebrio* MIP has been identified in *Drosophila*, except for Gp150, a protein involved in Notch signaling that has an aspartic acid-rich domain similar to that found in MIP.

In summary, microbial cell wall components of Gram-positive bacteria and fungi, such as PGN or β -1,3-glucan, are first recognized by specific recognition proteins, such as PGRP or β -1,3-glucan-recognition protein. Subsequently, the recognition signals trigger the activation of a serine protease cascade in which serine proteases are present as inactive zymogens, finally leading to the conversion of prophenoloxidase to phenoloxidase, resulting in the synthesis of melanin where melanization is required. The advantage of this insect prophenoloxidase cascade is that a minute microbial recognition signal is amplified by multiple activation steps. Until now, the *in vivo* importance of melanization during insect host defense has been intensively studied using classical biochemistry and molecular genetics. Currently, as the complete insect prophenoloxidase activation cascade gradually emerges at the molecular level, the predictions of pioneering researchers like Ohnishi and Ashida are turning out to be correct.

14.4.4.2. Melanin synthesis by parasite and parasitoid wasps

As described above, melanization is also essential for host defense against parasites and parasitoid wasps. In the mosquito *A. gambiae*, resistance to malaria parasites is deeply related to melanization of the malaria ookinete (Collins *et al.*, 1986). Following this discovery, many research groups, including the Kafatos group, intensively screened and identified a large set of regulators of melanization using microarray analysis and reverse genetics via RNAi; their findings suggest that melanization reactions are fine-tuned in response to malaria parasite infection (Sinden, 2004). Interestingly, Volz *et al.* (2006) reported that a refractory *A. gambiae*

strain easily synthesized melanin while under a chronic state of oxidative stress, explaining how this process leads to resistance to parasites. Now, using a highly diverse body of vector species and strains, many researchers are trying to understand the balance between parasite invasion and elimination by the mosquito innate immune system.

In early studies, biochemical evidence of dramatically reduced phenoloxidase activity was reported in lepidopteran larvae parasitized with different parasitoids (Sroka and Vinson, 1978). Beckage *et al.* (1993) first demonstrated that this inhibition effect could be mimicked by injection of *Cotesia congregata* bracovirus into non-parasitized larvae, but that inactivated virus had no effect, suggesting that downregulated phenoloxidase activity was caused by the bracovirus. Shelby *et al.* (2000) observed that the immunosuppressive *Campoletis sonorensis* ichnovirus (CsIV) reduces the protein level of several key enzymes in the melanin synthesis pathway, such as phenoloxidase, dopachrome isomerase and DOPA decarboxylase in parasitized fifth lepidopteran larvae, providing more evidence for the reduction of melanization in parasitized insects.

In the *Drosophila* system, phenoloxidase-deficient or DOPA-decarboxylase-deficient mutant flies are significantly compromised in their cellular immune responses, such as encapsulation of the endoparasite wasp *Leptopilina boucardi*, suggesting that melanization is important for host defense (Rizki and Rizki, 1990). Additionally, flies that were incapable of melanin synthesis because of the overexpression of serpin Spn27A in the hemolymph demonstrated compromised encapsulation cellular immunity and lowered ability to kill *L. boucardi* (Nappi *et al.*, 2005); this experiment demonstrated that the melanization reaction could be a target for innate immune suppression by parasitoid wasps. A recent interesting paper demonstrated that the parasitic wasp *Microplitis demolitor* injects a *Microplitis demolitor* bracovirus (MdBV) while laying its eggs in *M. sexta* larvae. MdBV secrete the serine protease inhibitor Egf1.0, which blocks melanization by competitively inhibiting *Manduca* PPAE-1 and -3, providing evidence of the importance of melanization for both the virus and its host wasps (Beck and Strand, 2007).

14.4.5. Immune Responses to Viral Infection

Insects are susceptible to highly diverse families of DNA and RNA viruses. Recently, many insect viruses have caused substantial damage to agriculture. For example, some insect viruses kill and threaten beneficial insects, such as the honeybee and silkworm. Some viral infections of insects, such as yellow fever virus, West Nile virus, and Dengue virus, can be transmitted to humans with severe side effects, including encephalitis (Mackenzie *et al.*, 2004). Clearly, insect models for studying host–virus interactions based on genetic and molecular biology will benefit society in many ways.

Genetic studies using *Drosophila* as a virus–host model system provide two types of innate immune responses to virus infection: a constitutive defense system that induces degradation of viral RNA by RNA interference (RNAi), and an inducible immune response that prevents viral infection by inducing a large number of genes after detecting viral particles (Kemp and Imler, 2009).

In 2005, the Imler group published pioneering work on *Drosophila* inducible responses to viral infection (Dostert *et al.*, 2005). They focused on one gene, *vir-1* (virus-induced RNA 1), which contains a consensus binding site for the transcription factor STAT92E. *Drosophila* C virus (DCV) infection triggers induction of STAT DNA-binding activity in fly nuclear extracts, indicating that the JAK (Janus Kinase)/STAT pathway may be involved in the induction of the *vir-1* gene. In *Drosophila*, the JAK/STAT pathway is composed of a single JAK kinase and a single STAT factor (known as STAT92E). The JAK kinase is also regulated by the cytokine receptor Domeless, which shows similarity to the gp130 subunit of the interleukin-6 receptor in mammals (Agaisse *et al.*, 2003). Analysis of the flies carrying a JAK kinase gene mutation showed a higher viral load than wild type flies, and succumbed more rapidly to DCV infection, indicating that some genes induced by DCV infection participate in the control of viral amplification. Based on these results, the Imler group proposed a model in which one cytokine of the Unpaired (Upd) family (Upd-1, -2, and -3) is induced by *Drosophila* C virus infection, and triggers an antiviral response to infected cells through activation of the JAK/STAT pathway (Sabin *et al.*, 2010) (Figure 2).

In the mosquito system, the Dimopoulos group demonstrated that the JAK/STAT pathway also restricts infection of Dengue virus, a medically important arbovirus (Souza-Neto *et al.*, 2009). They identified several genes that were upregulated by Dengue infection via the JAK/STAT pathway. Among them, the authors identified and partially characterized two JAK-STAT pathway-regulated and infection-responsive Dengue virus restriction factors (DVRFs) that contain putative STAT-binding sites in their promoter regions, suggesting that the JAK-STAT pathway is part of the *A. aegypti* mosquito's anti-dengue defense and acts independently of the Toll pathway and RNAi-mediated antiviral defenses.

In 1998, Fire and Mello discovered RNA interference (RNAi) (Fire *et al.*, 1998), revealing that double-stranded RNAs (dsRNAs) can be used as a tool to knock down specific genes. RNAi is a defense reaction based on the specific base-pairing between small host RNAs and invading pathogenic nucleic acids. RNAi is now an efficient biochemical tool for gene knockdowns and for examining important physiological functions in diverse biological processes. The first evidence of RNAi as an important antiviral defense mechanism came from plant studies (Li and Ding, 2001; Vance and Vaucheret, 2001).

Further studies showed that RNAi depends on small RNAs that are 21–30 nucleotides in length and are divided into three classes: small interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-associated interfering RNAs (piRNAs). Both siRNAs and miRNAs are known to be processed as duplexes from dsRNA precursors by an RNaseIII enzyme called Dicer (Hammond, 2005), but only after the primary miRNA transcripts (pri-miRNA) that contain imperfect intramolecular stem-loops are first processed within the nucleus. The resulting precursor miRNAs (pre-miRNA) are then converted into a single mature miRNA species in the cytoplasm (Bartel, 2004). In contrast to siRNAs and miRNAs, piRNAs are about 30 nucleotides in length and are found in the germ-line of flies and vertebrates, which are Dicer-independent (Zamore, 2007). The *Drosophila* genome has two Dicer enzymes, Dicer-1 and Dicer-2, which produce two types of regulatory RNA: microRNAs (miRNAs) and small interfering RNAs (siRNAs). Dicer-1 induces the processing of the precursor miRNAs to mature miRNAs, whereas Dicer-2 recognizes long dsRNAs and cleaves them into siRNAs (Lee *et al.*, 2004).

Drosophila studies related to RNAi immune responses against viral infection began with analysis of Dicer-2 mutant flies. These flies showed increased susceptibility and lethality to RNA virus infections from DCV, Cricket paralysis virus, Flock House virus, and *Drosophila* X virus (Galiana-Arnoux *et al.*, 2006; Wang *et al.*, 2006). An increased viral load and high levels of viral RNA were observed in these virally infected mutant flies, suggesting that RNAi is a crucial defense mechanism for viral infection. Additionally, the detection of siRNAs corresponding to viral sequences in infected cells strongly supported a mechanism whereby dsRNA, corresponding either to the viral genome or to replication intermediate forms, is detected by Dicer-2 and cleaved into siRNAs. These data also demonstrated that the Dicer-2-mediated siRNA pathway is a highly specific antiviral defense system based on the pairing of complementary nucleic acids. While Dicer-2 was suggested to play an essential role in the RNAi pathway, Imler recently reported that Dicer-2 also induces triggering of a downstream antiviral signaling cascade upon binding and recognition of viral dsRNA. This cascade results in the induction of the *Vago* gene, an antiviral gene that is required to restrict viral replication in flies (Deddouche *et al.*, 2008). *Vago* expression was shown to be dependent upon Dicer-2. Since Dicer-2 belongs to the DExD/H-box helicase family, as do the RIG-I-like receptors (Takeuchi and Akira, 2008) involved in sensing viral infection and mediating interferon induction in mammals, the authors proposed that this family represents an evolutionarily conserved sensor molecule that can detect viral nucleic acids and regulate antiviral responses.

Completely unique molecules are also identified in the hemolymph of virus-infected flies (Sabatier *et al.*, 2003). After injection of DCV into the *Drosophila* thorax, the induced molecules were analyzed by MALDI-TOF mass spectrometry and compared with those produced after the injection of bacteria or fungal spores. Interestingly, pherokine-2 (Phk-2), which was previously characterized as a putative odor/pheromone binding protein, was specifically induced, suggesting that host-defense mechanisms against viral infection are different from the mechanisms operating against bacterial or fungal infections in flies.

Taken together, these recent studies highlight the role of RNAi and innate immune signaling in antiviral defense in insects. However, many questions remain. For example, the real biological functions of only a few virus-specific inducible molecules, such as *Vago* and DVRF, have been elucidated. It is also unknown how invading viruses are recognized by the host. The RNAi machinery recognizes dsRNA, but the molecular mechanism is unclear. Finally, the known antiviral defense responses do not yet span the whole of host antiviral defense; loss-of-function mutation studies showed only modest impact on survival and viral replication, suggesting the presence of unidentified antiviral responses in the host. Further studies are required to identify new effector molecules and signaling pathways in insect antiviral defense.

14.4.6. Immune Responses to Malaria Infection

Malaria is a widespread and devastating vector-borne disease. *Plasmodium*, the parasite responsible for malaria, is transmitted to human beings through the blood of female *Anopheles* mosquitoes. Similar to other vector parasitic systems, *Plasmodium* induces multi-step developmental transformations inside the mosquito vector during infection of the host. The life cycle of parasites in mosquitoes can be summarized as follows. After infecting the blood, the fusion of male and female gametes generates a diploid motile zygote (ookinete), which rapidly invades epithelial cells and, upon reaching the basal side of the midgut, transforms into an oocyst. Two weeks later, the sporogonic oocyst releases thousands of newly formed sporozoites that migrate and invade the salivary glands. The life cycle of *Plasmodium* parasites in mosquitoes ends when the salivary gland sporozoites are injected via the saliva fluid into the next host during feeding (Sinden, 2002).

Two methods are commonly used to control malaria: (1) anti-malarial agents, and (2) controlling mosquito populations using entomological approaches. Anti-malarial drug treatments are only useful for malaria-infected patients. However, a mosquito population can be reduced using insecticides or water management. In addition, prevention of mosquito exposure using bed nets and repellent chemicals greatly limits the spread of malaria. As a new approach, vector control methods have been developed

from molecular studies of mosquito innate immune responses to *Plasmodium* parasites. Here, we summarize the current knowledge of the mosquito innate immune responses to *Plasmodium* infection.

14.4.6.1. Toll, IMD, and JAK/STAT signaling pathways after *Plasmodium* infection In the mosquito, the sequence complexity and redundancy of the Toll pathway make it difficult to predict the exact biological function of mosquito Toll orthologs using comparative genomics. For example, the *Anopheles* genome encodes 10 Toll receptors, four of which are orthologs of *Drosophila* Toll receptors. However, four other genes are orthologs of the *Drosophila* Toll-1 and Toll-5 receptors. In addition, at least six *Anopheles* Spätzle genes are similar to those of *Drosophila*, but the phylogenetic relationship of these 12 genes is complex. Another pathway that is different from that of *Drosophila* is the Toll receptor-dependent regulation of NF- κ B/Rel transcription factors. Dorsal is a major regulator of *Drosophila* developmental processes, and Dif is essential for innate immune responses. However, *Anopheles* do not encode the Dif gene. The mosquito homolog of Dorsal is Gambif1 (also referred to as Rel1). Upon bacterial infection, Rel1 translocates to the nucleus within the mosquito fat body, and may regulate the immune system (Barillas-Mury *et al.*, 1996).

The intracellular counterparts involved in the *Anopheles* Toll receptor signaling display high homology with those of *Drosophila*, such as MyD88, Tube, Pelle, and Cactus. *Drosophila* MyD88 and Tube contain Toll and IL-1R (TIR) domains, suggesting that these proteins may interact. Pelle contains an additional Ser-Thr kinase domain within its death domain. Cactus contains an ankyrin-repeat domain, and is a negative regulator of the Toll pathway, inhibiting the nuclear translocation of NF- κ B/Rel1 proteins. The biological functions of these *Anopheles* counterparts were primarily elucidated by RNAi experiments.

In contrast, the *Anopheles* IMD pathway splits into two signaling branches. One branch is similar to the mammalian C-Jun/JNK pathway and activates the transcription factor AP-1, whereas the other branch activates NF- κ B, leading to the expression of the transcription factor Rel2 (corresponding to *Drosophila* Relish). Rel2 exists as two forms in mosquito cells; Rel2-S lacks the inhibitory ankyrin domain, and full-length Rel2-F is inactive until an immune response is triggered. Similar to Cactus in the Toll pathway, Caspar functions as a negative regulator of the *Anopheles* IMD pathway. Caspar, a Fas-associated homolog, was initially identified as a negative regulator of Relish activation in *Drosophila* (Kim *et al.*, 2006). Recent studies had indicated that the *Anopheles* IMD pathway is more specific to malarial infection in comparison to the Toll pathway (see below).

As the third major immune pathway, JAK/STAT signaling is important for antiviral immunity in *Drosophila* (see above), and *Drosophila* gut immunity (see below). Two STAT transcription factors (STAT-A and STAT-B) have been identified in the *A. gambiae* genome, whereas only one STAT is present in *Drosophila*. STAT-B regulates the transcription of STAT-A, which is the predominant form expressed in adult mosquitoes. Although translocation of STAT-A into the nucleus is suggested to upregulate the expression of anti-plasmodium effectors, the detailed molecular mechanism is still obscure.

14.4.6.2. Effector molecules against *Plasmodium* infection Many *A. gambiae* immune-responsive genes have been identified by intensive examination of expression profiles from RNAi-mediated gene silenced mutant mosquitoes, which are infected with bacteria, fungi, and malaria parasites (Dimopoulos *et al.*, 1998). Among them, thioester-containing protein1 (TEP1), leucine-rich repeat immune protein 1 (LRIM1), *Anopheles Plasmodium*-responsive leucine rich repeat 1 (APL1), C-type lectin 4 (CTL4), Serpin 2 (SRPN2), and fibrinogen-related proteins (FREPs) are suggested to be major regulatory molecules affecting *Plasmodium* development (Cirimotich *et al.*, 2010).

TEP1 belongs to a family of thioester-containing proteins, and is homologous to the vertebrate factors C3/C4/C5 and to members of the α 2-macroglobulin family. In *A. gambiae*, TEP1 is constitutively secreted from mosquito hemocytes and is present in the hemolymph as a 165-kDa zymogen. TEP1 is processed into an 80-kDa fragment upon parasite infection (Levashina *et al.*, 2001). Levashina and colleagues have thoroughly studied this molecule, and suggest that TEP1 functions as an opsonin on the surface of parasites that triggers immune responses.

LRIM1 and APL1 are leucine rich repeat (LRR) domain-containing proteins. Recent studies demonstrated that LRIM1 and APL1C form a disulfide-linked, high molecular weight complex that is secreted into the *Anopheles* hemolymph. This heterodimeric complex interacts with TEP1, leading to the cleavage and activation of TEP1. Therefore, LRR domain-containing proteins play a key role in mediating anti-*Plasmodium* immunity in mosquitoes. More than 20 LRIM1-like proteins have been identified from several mosquito genomes, but no orthologs have been identified in other organisms, indicating that these genes are mosquito-specific (Povelones *et al.*, 2009). In addition, Osta *et al.* (2004) demonstrated that LRIM1 and APL1 play an important role in parasite melanization and killing during early-stage *P. berghei* infection.

The CTL family is the most diverse animal lectin family. CTLs bind carbohydrates in a Ca²⁺-dependent manner through the C-terminal carbohydrate recognition domain. In the *A. gambiae* genome, 23 genes that encode C-type lectin domains have been identified. CTL4 and

CTLMA2 are agonist molecules of the rodent *Plasmodium* parasite, and silencing of either of these genes induces massive melanization of *P. berghei* ookinetes in the mosquito midgut epithelium, inhibiting mosquito development at the pre-oocyst stage (Osta *et al.*, 2004). However, CTLs do not seem to be involved in defense responses to human *Plasmodium* parasites.

SRPN is a member of a large family of serine protease inhibitors that are present in all higher eukaryotes, as well as in some viruses. In *Anopheles*, Michel *et al.* (2005) reported that knockdown of SRPN2 triggers spontaneous melanization of mosquito blood cells and reduces the life-span of adults. Depletion of SRPN2 increased parasite killing and stimulated melanization.

Fibrinogen-related proteins (FREPs) contain a fibrinogen-like domain, which is evolutionarily conserved from invertebrates to mammals. FREP genes are present in mosquitoes, and are significantly more expressed in *A. gambiae* (58 genes) in comparison to *Ae. aegypti* (37 genes) and *D. melanogaster* (14 genes). RNAi-mediated gene silencing showed that FREPs in *Anopheles* are involved in anti-*Plasmodium* defense. For instance, FBN39 specifically protected mosquitoes against *P. falciparum* infection (Christophides *et al.*, 2002).

14.4.6.3. Regulation of anti-malarial immune responses in mosquitoes Elucidation of the molecular mechanisms of the mosquito innate immune response to *Plasmodium* infection is expected to help discover novel malaria control strategies. Many researchers are trying to determine how mosquito Toll and IMD immune signaling pathways mediate anti-*Plasmodium* responses.

In this respect, two breakthrough studies have been performed using RNAi-mediated Cactus-silenced and RNAi-mediated Caspar-silenced adult female mosquitoes (Frolet *et al.*, 2006; Garver *et al.*, 2009). These studies enabled us to understand mosquito resistance mechanisms and how malarial ookinetes are killed by host mosquitoes. *Anopheles* Cactus and Caspar proteins are specific negative regulators of *Anopheles* Toll and IMD signaling pathways, respectively. In the first report, Frolet *et al.* (2006) introduced a novel concept of mosquito-malaria immunity referring to a “pre-invasion or basal immunity” phase and “post-invasion or induced immunity” phase. Basal immunity is constitutive and induces the production of immune factors, including TEPI, which is dependent on the two NF- κ B factors, Rel1 (Dif-like) and Rel2 (Relish-like). When TEPI and other hemocyte-derived immune factors are depleted after a 24-h infection, immune responses are activated and induce the transcription of genes encoding multiple immune factors. It was also noted that the replenishment of depleted factors was both NF- κ B-dependent and -independent. Another remarkable discovery is that boosting basal immunity by RNAi-mediated silencing of Cactus was sufficient to completely block

P. berghei development to the oocyst stage, suggesting that Cactus is capable of complete elimination of malarial parasites in mosquitoes. These authors developed a powerful system to determine unique mosquito proteins involved in parasite killing, which can be used to identify resistant mosquito strains.

In the second report, Garver *et al.* (2009) focused on the biological effects of Caspar during malarial infection and transcriptional regulation of three antiplasmodium genes, TEPI, LIRM1, and FREP9, using an RNAi-mediated Caspar-silenced mosquito. The authors obtained several intriguing results: (1) Caspar-silenced *A. gambiae* is refractory to the natural virulent human malaria parasite, *P. falciparum*; (2) depletion of Cactus or Caspar reduced the number of oocysts in the midgut, indicating that both the Toll and IMD immune pathways are involved in the defense against *P. falciparum* and *P. berghei*, albeit to varying degrees depending on the parasite species; and (3) silencing of *A. albimanus* and *A. stephensi* Caspar genes generated a refractory phenotype regardless of the *Anopheles* species.

These studies are significant because they implied the possibility for the development of malaria control strategies based on mosquito innate immunity. Namely, rather than targeting the malaria parasite, the mosquito host can be targeted by upregulating conserved molecules to enhance basal immunity. In addition, the differences between immunity-based resistance to rodent and human malaria parasites are dependent on the transcription factor Rel. For example, Rel2-based immunity is most efficient against *P. falciparum*, whereas Rel1-based immunity is most efficient against *P. berghei*.

14.5. Cellular Innate Immune Responses

14.5.1. Phagocytosis

Phagocytosis is a major cellular innate immune response in both mammals and insects. This process is essential for various biological events, including the elimination of pathogenic microbes, removal of apoptotic cells, tissue remodeling, and induction of innate and adaptive immune responses. Major insect hemocytes are classified as crystal cells, lamellocytes, and plasmatocytes. Plasmatocytes control phagocytic elimination of invading microbes. During the past two decades, three major questions have been addressed with regard to the molecular mechanism of insect phagocytosis: (1) What kind(s) of receptors are involved in the induction of insect phagocytosis? (2) What is the ligand molecule that activates phagocytosis? and (3) What humoral factors in the insect plasma promote the phagocytosis?

Intensive biochemical and functional studies of phagocytic receptors, ligands, and intracellular signaling pathways have been performed in mammalian systems (Jutras and Desjardins, 2005). However, in *Drosophila*, six

receptor molecules involved in phagocytosis have been characterized: Croquemort, *Drosophila* scavenger receptor (dSR-CI), PGRP-LC, Draper, Eater, and Nimrod C1 (Stuart and Ezekowitz, 2008).

Croquemort (catcher of death) is a member of the CD36 family of proteins, which is specifically expressed in *Drosophila* macrophage-like hemocytes and is involved in the clearance of apoptotic cells in *Drosophila* embryos (Franc *et al.*, 1999). Human CD36 is a macrophage scavenger receptor that is able to bind a subset of polyanionic ligand molecules. Genetic analysis demonstrated that Croquemort is essential for apoptosis but is not required for the engulfment of bacteria. This suggests that signals generated by dying cells increase the expression of Croquemort, which could facilitate the clearance of cell corpses. *Drosophila* Peste, which was identified by an RNAi screen, is a class B scavenger receptor and is required for the uptake of mycobacteria, but not *E. coli* or *S. aureus* (Philips *et al.*, 2005), suggesting that mammalian class B scavenger receptors may be involved in the recognition of mycobacteria by insects.

Drosophila scavenger receptor C1 (dSR-C1) is a multidomain modular protein consisting of a 609-residue membrane protein containing several well-known sequence motifs, including two complement control protein (CCP) domains, a somatomedin B domain, a MAM (a domain found in Meprin, A5 antigen) domain, and RPTP Mu and mucin-like domains. R  met *et al.* (2001) demonstrated that dSR-C1 is a receptor that recognizes both Gram-negative and Gram-positive bacteria, but not yeast. This receptor binds a wide variety of ligands. In addition, dSR-CI does not appear to be required for the *Drosophila* AMP response. Even though the ligand molecules in these bacteria have not been determined, scavenger receptors are primordial pattern recognition molecules that mediate evolutionarily conserved innate immunity.

Drosophila PGRP-LC regulates DAP-type PGN-mediated IMD signaling. Initially, it was discovered as a phagocytosis receptor by R  met *et al.* (2002). Using a dsRNAi-based screen in *Drosophila* macrophage-like cells, 34 genes involved in phagocytosis were identified. Of them, PGRP-LC is involved in phagocytosis of Gram-negative but not Gram-positive bacteria, demonstrating that *Drosophila* PGRP-LC is an essential molecule for the recognition and signaling of Gram-negative bacteria during the insect innate immune response.

Draper (Manaka *et al.*, 2004), Eater (Kocks *et al.*, 2005), and Nimrod C1 (Kurucz *et al.*, 2007) are receptor molecules containing epidermal growth factor (EGF) repeats. Similar proteins are found in silkworms and the beetle *H. diomphalia* (Ju *et al.*, 2006), in which they function as secreted opsonins and are involved in bacterial clearance. The EGF-like motifs have a conserved consensus sequence CxPxCxxx-CxNGxCxx PxxCxGxxGY, and are separated by variable

loops of typically 6–11 residues. This motif differs significantly from the typical EGF repeat (xxxxCx2-7Cx1-(G/A)xCx1-13ttaxCx-CxxGax1-6GxxCx).

Draper was identified as a *Drosophila* homolog of the *C. elegans* CED-1 protein, and has 15 EGF repeats. It is involved in hemocyte phagocytosis of apoptotic cells, but not of zymosans. Draper acts as a receptor in the phagocytosis of apoptotic cells by hemocytes and glial cells in *Drosophila* embryos. Although apoptotic *Drosophila* cells express the membrane phospholipid phosphatidylserine, the best characterized eat-me signal found in mammals, Draper, does not recognize phosphatidylserine. Further studies into the intracellular signaling and identification of Draper ligand molecules have been performed (see below). Eater, a cell surface receptor containing a 32 EGF repeats motif, regulates the phagocytosis of *E. coli* and *S. aureus*, suggesting that Eater is a major phagocytic receptor for a broad range of bacterial pathogens. As expected, Eater was expressed in the plasmatocytes and the lymph glands, but not in the crystal cells, lamellocytes, or the fat body, which are cells and organs not involved in phagocytosis. Biochemical data demonstrated that the N-terminal domain of Eater directly binds to acetylated and oxidized low-density lipoproteins, which act as mammalian scavenger receptor ligands.

Nimrod C1 (NimC1) is also involved in the phagocytosis of bacteria. This receptor is a 90- to 100-kDa transmembrane protein with 10 EGF repeats, similar to Draper and Eater. Furthermore, suppression of NimC1 expression by RNAi inhibited the phagocytosis of *S. aureus* in plasmatocytes. However, when NimC1 was overexpressed in S2 cells, phagocytosis was induced in response to *S. aureus* and *E. coli*. Interestingly, the *NimC1* gene is part of a cluster of 10 related *Nimrod* genes on chromosome 2 in *Drosophila*, and similar clusters of *Nimrod*-like genes were conserved in other insects, such as *Anopheles* and *Apis*. Although these five receptors were discovered as *Drosophila* phagocytosis receptors, how these receptors regulate intracellular pathways directing cytoskeletal remodeling and membrane trafficking was not addressed. In addition, the identification of phagocytic receptor ligands is essential to understanding the molecular mechanism of phagocytosis.

In 2009, the Nakanishi group identified an endoplasmic reticulum protein, Pretaporter, as a ligand for Draper (Kuraishi *et al.*, 2009). Pretaporter appeared to relocate from the endoplasmic reticulum to the cell surface during apoptosis to serve as an eat-me signal. Pretaporter-bound Draper was tyrosine phosphorylated, and required the adaptor Ced-6 and the small G-protein Rac to signal, suggesting that Pretaporter activates the engulfment pathway involving Draper/Ced-6/Rac, which is reminiscent of the CED-1/CED-6/CED-1 pathway in *C. elegans*. In addition, Calreticulin, another endoplasmic reticulum protein in *Drosophila*, acts as an eat-me signal (Kuraishi *et al.*, 2007);

however, a corresponding phagocytosis receptor has yet to be identified. Subsequently, the Nakanishi group attempted to identify the ligand molecule that is recognized by *Drosophila* hemocytes. Analysis of the cellular immune response using a series of *S. aureus* mutants that are defective in cell wall synthesis and *Drosophila* phagocytosis identified lipoteichoic acid as the ligand for Draper (Hashimoto *et al.*, 2009). Phagocytic receptors activate uptake machinery during phagosome maturation. Recent genome-wide RNAi screening studies in *Drosophila* S2 cells have identified proteins required for microbe uptake (Rämet *et al.*, 2002; Stuart *et al.*, 2007). After recognition of the ligand by phagocytic receptors, curvature of the membrane and extension of pseudopod tips are necessary for phagocytosis. RNAi-based analysis revealed that membrane curvature is regulated by coat proteins containing Clathrin and a coatamer protein complex. The properties of the coatamer protein complex are not discussed in detail here, as this is extensively covered in other reviews (for example, Stuart *et al.*, 2007). To internalize target microbes more efficiently, it is necessary for phagocytes to recruit additional membranes from intracellular components, such as endosomes and the endoplasmic reticulum. During this process, the exocyst, an octodimeric complex, tethers the recruited endosomes to the phagocytotic cup. These exocyst and coatamer protein complexes are thought to regulate phagosome maturation. However, because *Drosophila* phagocytosis is complicated by many cell surface receptors and key machinery components are partially redundant, studies dissecting the complexity of ligand recognition and intracellular signaling are needed. In addition, because the biological significance of insect phagocytosis is unclear, further studies addressing the diverse cellular fate of internalized targets and the biological functions of matured phagosomes are also needed.

14.5.2. Hemocyte Hematopoiesis during Wasp Parasitism

As described above, early studies demonstrated that *Drosophila* plasmocytes are involved in phagocytosis, and crystal cells are required for melanization. However, lamellocytes are responsible for encapsulation (Meister and Lagueux, 2003). Encapsulation, another cellular defense response, occurs when foreign bodies that are too large to be phagocytosed are surrounded. Morphological analysis of encapsulation suggested that blood cells attach to the invading foreign body, such as wasp eggs, and establish a multi-layered cellular capsule. When parasitoid wasps lay their eggs in *Drosophila* larvae, differentiated lamellocytes, which do not exist in naïve conditions, induce encapsulation. This observation has led to questions about whether the blood cells differentiate from larval hematopoietic progenitors (prohemocytes) in response to wasp

infection, and how blood cell homeostasis is regulated at the molecular level. These questions are fundamental to the elucidation of insect encapsulation during cellular immune responses.

Similar to the recent development of mammalian stem cell research, *Drosophila* hemocyte hematopoiesis research has quickly developed (Crozatier and Meister, 2007). Here, we overview studies defining the relationship between hemocyte hematopoiesis and wasp infection. In 1984, Rizki reported that massive proliferation of lamellocytes occurred in the lymph upon parasitism of wasp parasitoid, while larvae maintained a balance of 95% plasmatocytes and 5% crystal cells (Rizki and Rizki, 1984). Subsequently, Sorrentino *et al.* (2004) observed that proliferation of lamellocytes was dependent on JAK/STAT activity, and Krzemien *et al.* (2007) reported that the JAK/STAT pathway is required for maintenance of the lymph gland and the control of hemocyte production in third instar larvae. In addition, JAK/STAT signaling is turned off upon wasp infestation, resulting in differentiation of prohemocytes into lamellocytes. The JAK/STAT signaling pathway is evolutionarily conserved, and mammalian genomes encode multiple components of the JAK/STAT pathway, including multiple receptor subunits. Therefore, it is not easy to define the biological functions of each component and receptor. In contrast to mammals, only one receptor (Domeless), one JAK (Hopscotch), one STAT (Stat92E), and three cytokines – Unpaired 1 (Upd1), Upd2, and Upd3 – have been identified and characterized in *Drosophila*, indicating that it is easy to determine the molecular mechanism because of its simplicity.

In 2010, Makki *et al.* (2010) reported intriguing results regarding the regulatory mechanism of the JAK/STAT pathway. A novel negative regulator that functions as a switch-off molecule in this pathway was identified. They noticed one gene, *CG14225/lat*, coded for a protein structurally related to the Domeless receptor, that had extracellular domains similar to the cytokine-binding domain (CBM) and Lat-Domeless Homology Region (LDHR). The *lat* gene is selectively expressed in hematopoietic progenitors in the *Drosophila* lymph gland. Several novel findings regarding *lat* gene function were made: (1) *lat* mutant flies had a dysfunctional cellular immune response to wasp parasitism; (2) Lat protein functioned as a switch-off molecule of the JAK/STAT signaling pathway by heterodimerizing with Domeless *in vivo*; (3) Upd3, but not Upd1 and Upd2, activated the JAK/STAT signaling pathway; and (4) wasp parasitism increased the Lat/Dome protein ratio, decreasing JAK/STAT signaling and differentiating prohemocytes into lamellocytes. These results suggest that the *Drosophila* cellular immune response to wasp infection is regulated by two molecules: Lat and Domeless. In addition, the JAK/STAT pathway is inactivated by an increased Lat/

Domeless protein ratio, upon wasp parasitism *in vivo*. Therefore, this study identifies the need to determine whether the Lat-like short, non-signaling receptor homolog can function as an antagonist of the mammalian JAK/STAT signaling pathway.

14.6. Newly Emerging Topics in Insect Immunology

14.6.1. Gut Insect Immunology

The insect gut contains various microorganisms, such as symbiotic commensal and food-borne pathogenic bacteria. The survival of symbiotic gut microbes is achieved by evolutionary positive selection. It is unknown how the insect gut epithelium tolerates commensal bacteria while maintaining the ability to trigger an immune response upon pathogenic microbe infection. The basis for the symbiotic relationship between the insect gut epithelia and symbiotic microbes is also unknown. To answer these questions, *Drosophila* was used as a model system because the *Drosophila* gut is known to harbor approximately 10–20 bacteria species in the midgut region, whereas the human gut is thought to host 500–1000 bacteria species. Taking advantage of this simplicity, several groups have begun to elucidate the molecular mechanisms underlying innate immunity homeostasis in the *Drosophila* gut. These studies concluded that *Drosophila* gut immunity primarily relies on two effector molecules, AMPs and reactive oxygen species (ROS), and that these two molecules are tightly regulated to prevent the growth and proliferation of pathogenic bacteria. However, AMPs and ROS preserve commensal bacteria within the gut. Lee and colleagues reported seminal work regarding insect gut immunity (Ha *et al.*, 2005a; 2005b, 2009a, 2009b; Ryu *et al.*, 2008). Here, we summarize recent research progress in insect gut immunity.

14.6.1.1. NF- κ B-AMP-mediated gut immunity An intriguing aspect of gut immunity is that constant direct contact between gut epithelia and commensal bacteria induces the activation of a constitutive basal immune response, suggesting that AMPs are always produced, even in the presence of commensal bacteria. Several groups reported that the basal level of AMP in *Drosophila* epithelia is dependent on the IMD pathway and independent of the Toll pathway. However, it was unknown how insects accommodate commensal bacteria in spite of the presence of AMP, and how insects inhibit the growth and proliferation of pathogenic bacteria in gut.

Recently, Lee and colleagues elegantly demonstrated that *Drosophila* control gut homeostasis through Caudal (Cad). Cad, a homeobox transcription factor, was originally discovered as a regulator of the antero-posterior body axis in *Drosophila* (Dearolf *et al.*, 1989). Cad

expression is tightly regulated in response to developmental signals during embryogenesis. However, Cad expression is altered in the intestine and Malpighian tubules after embryogenesis. Lee and colleagues found that *Drosophila* AMPs, such as diptericin and cecropin, which are expressed by a Relish-dependent IMD pathway in the fat body, were strongly inhibited at the transcriptional level by Cad. This occurred in spite of the basal level of IMD signaling (Ryu *et al.*, 2008), suggesting that Cad acts as a gut-specific repressor of AMP gene expression. Based on these results, they hypothesized that repression of NF- κ B-dependent AMP production is required to preserve and tolerate commensal bacteria, and that regulation of constitutive AMP expression leads to modification of the commensal community structure in gut (Figure 4).

To address these hypotheses, changes in the bacteria population after manipulation of AMPs were studied. When AMP genes were overexpressed, the expression of major commensal bacterium (A911 strain of *Acetobacteraceae*) decreased; however, the amount of minor commensal bacterium (G707 strain of *Gluconoacetobacter*) increased. These surprising results were confirmed by *in vitro* analysis; the A911 strain was very sensitive to synthetic cecropin, whereas the G707 strain was relatively resistant. These results proposed that, in the presence of the A911 strain, AMP activation antagonizes the dominance of the G707 strain under normal conditions. However, upon AMP overexpression, the A911 population is increased more than that of the G707 strain. In addition, the G707 strain modified the commensal bacterial community found in Cad-knockdown flies, leading to severe apoptosis in the gut (Ryu *et al.*, 2008). Furthermore, gut pathology of Cad-knockdown flies was attenuated in the absence of commensal bacteria. Finally, when the G707 strain was introduced into the gut of germ-free flies in the absence of the A911 strain, apoptosis of intestine cells and host mortality were induced, suggesting that outgrowth of the G707 strain is deleterious to the host.

Along with Lee's studies, several other research groups identified several novel molecules that function as negative regulators of IMD-mediated Relish activation in gut epithelia. PGRP domain-containing proteins, such as PGRP-SC and -LB, degrade DAP-type PGN. These PGRPs are thought to prevent excessive IMD pathway activation by sustaining a low level of PGNs in the gut (Bischoff *et al.*, 2006; Zaidman-Remy *et al.*, 2006). Another group identified an antagonist of the PGRP-LC receptor, PIMS (Rudra; PIRK), which inhibits IMD signaling (Lhocine *et al.*, 2008). These studies identified negative regulatory molecules that work together to regulate Relish activity in gut epithelial cells. In addition, because IMD-mediated Relish activation modifies the basal expression of negative regulators, such as PGRP-SC, -LB and PIMS, commensal bacteria-mediated and IMD-mediated Relish activation is suggested to

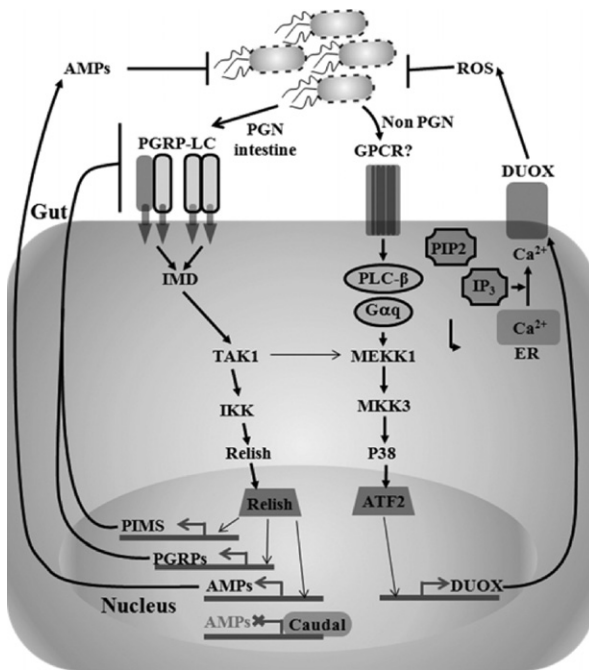


Figure 4 Proposed *Drosophila* gut immune signaling network. Two different effector molecules, AMP and ROS, are produced by activation of the Relish-mediated IMD pathway and DUOX-mediated signaling pathway, respectively. When excessive bacterial PGNs are released onto the gut epithelial cell surface following serious bacterial infection, large amounts of AMPs are produced after strong activation of the Relish-mediated IMD pathway. The excessive AMP production is negatively regulated by Caudal expression. Simultaneously, non-PGNs-mediated DUOX transcription is upregulated via p38-mediated ATF2 activation. This p38 activation is regulated by a PGNs-independent PLC- β pathway and a PGNs-dependent IMD pathway that merge into MEKK1. The production of large amounts of DUOX enzyme after upregulation of DUOX gene produces large amounts of ROS, which can kill bacteria in the gut. In contrast, small amounts of PGNs are released from commensal bacteria under uninfected conditions. In these conditions, p38 is in an inactive state via a PLC- β -dependent feedback loop in order to prevent excessive *Duox* expression. Also, PGRP-LC-mediated AMP production is regulated by Caudal, resulting in proper maintenance of the gut microbial population. Activation of the IMD pathway is negatively regulated by catalytic PGRPs and PIMS. Abbreviations: ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; IKK, I κ B kinase; IP3, inositol-1,4,5-trisphosphate; PIP2, phosphatidylinositol-4,5-isphosphate.

play a pivotal role in a negative regulatory feedback loop (**Figure 4**). Taken together, gut immunity studies using *Drosophila* as an animal model enabled the study of the normal flora community, which prevents colonization of potentially pathogenic microbes. Clinically, it was suggested that an imbalance in the equilibrated gut flora of humans induced by antibiotics, or anti-cancer chemotherapies or radiation, can lead to pathogenic infections (Sommer *et al.*, 2009).

14.6.1.2. Duox- and ROS-mediated gut immunity

As described above, NF- κ B-AMP-mediated gut immunity is primarily regulated by DAP-type PGNs, which are secreted from commensal or pathogenic bacteria. However, recently it has been discovered that PGN-independent generation of ROS regulates insect gut immunity homeostasis. ROS are a by-product of the metabolism of oxygen, and have biologically important roles in immunity and cell signaling. They are known to have bactericidal effects due to their chemically reactive properties. The bactericidal activity of ROS in innate immunity has been well studied in mammalian phagocytes (Leto and Geiszt, 2006). NADPH oxidase within the phagosome (NOX2) produces superoxide from molecular oxygen. In a study of ROS-mediated insect gut immunity, Ha *et al.* (2005b) showed that flies lacking immune-regulated catalase (IRC) are highly susceptible to gut infection. In addition, they demonstrated that the mortality of IRC-deficient flies was due to oxidative stress after exposure to microbial components, rather than to the overproliferation of ingested microbes, suggesting that the insect gut may have a novel ROS generating system that is responsive to microbial components, but not PGN. They hypothesized that ROS generated from insect gut epithelia cells, which are not traditional phagocytic cells, act as bactericidal effector molecules in gut immunity.

Subsequently, Ha *et al.* (2005a) discovered that Duox, an oxidase, is essential for the regulation of gut immunity. *Drosophila* have one NOX and one Duox gene in their genome. Genetic analysis demonstrated that *Drosophila* Duox, but not NOX, is involved in microbial clearance in the gut, and that Duox knock-down flies are highly susceptible to gut infection. This susceptibility is due to the overproliferation of ingested microorganisms. How Duox is regulated after recognition of non-PGN molecules of gut microbes is unknown. As shown in **Figure 4**, Ha *et al.* (2009a, 2009b) provided evidence that unidentified non-PGN components of bacteria are involved in Duox-mediated ROS generation *in vitro* and *in vivo*. Subsequently, they determined how Duox is regulated. A non-PGN ligand molecule was recognized by an unknown membrane-associated G protein-coupled receptor (GPCR) and the ligand recognition signal was transmitted to the intracellular downstream molecules, G α q and phospholipase C β (PLC β), leading to the mobilization of intracellular calcium via generation of inositol 1,4,5-trisphosphate (IP3). This PLC β /IP3-mediated calcium mobilization is sufficient for the spontaneous activation of Duox and subsequent ROS generation to promote bactericidal activity. Furthermore, G α q and PLC β -deficient mutant flies were unable to control gut microbes, and were highly susceptible to infection under conventional rearing conditions. However, this susceptibility was rescued under germ-free rearing conditions,

confirming that regulation of Duox activity is a crucial factor in insect host defense.

Next, these authors asked how *Duox* gene expression is transcriptionally regulated in response to infectious conditions, and what kinds of intracellular molecules are involved in the regulation of *duox* gene expression. They found that *Duox* gene expression was regulated by a MEKK1–MKK3–p38 pathway in the presence of a high concentration of microbial ligands, which contain both PGN and non-PGN molecules. This result was unexpected, because PGN induced *Duox* gene expression but not Duox enzyme activity. These results clearly demonstrate that activation of the MEKK1–MKK3–p38 pathway occurs both in a PGN-independent manner via PLC β and a PGN-dependent manner involving the PGRP-LC and IMD pathways (Figure 4).

Because ROS is also harmful to host cells, ROS generation is tightly regulated. Lee and colleagues demonstrated that Duox expression was precisely regulated by cross-talk between a PGN-PGRP-LC-dependent IMD–MEKK1–MKK3–p38–ATF2 signaling pathway and a non-PGN-GPCR-dependent PLC β -signaling pathway. Namely, in the presence of the normal commensal gut population, Duox enzyme-mediated constitutive activation resulted in low PLC β activity and downregulated PGN-dependent activation of p38. This suggests that the basal expression of Duox is required to reduce oxidative damage during the commensal–gut epithelia interaction. Conversely, upon infection by pathogenic bacteria, cooperation of Duox enzyme activity is required, and leads to ROS production. In this situation, PGN-independent PLC β signaling and PGN-dependent PGRP-LC-mediated IMD signaling activate MEKK1 to induce p38-dependent Duox induction. At the same time, activation of PLC β by high concentrations of non-PGN ligands increases Duox enzymatic activity (Figure 4). These data demonstrate how the host achieves gut microbe homeostasis by efficiently combating pathogenic bacteria while at the same time tolerating commensal microbes in the gut.

Finally, even though recent data have elucidated how insects regulate gut immunity, there are several questions that are the focus of future studies. First, how do insect gut cells distinguish between commensal bacteria and pathogenic bacteria? Second, what is the ligand structure capable of activating Duox? Future studies will enable a better understanding of the molecular mechanism of insect gut immunity.

14.6.2. Immune Priming of Insect Immunology

Insects are considered to lack both immune specificity and immune memory (immune priming) because they do not possess an adaptive immune system. However, recent molecular and genetic studies of fruit flies and mosquitoes

have identified diverse immune receptors in invertebrates (Dong *et al.*, 2006; Watson *et al.*, 2005), providing an unexpectedly high degree of specificity and immune memory within invertebrate systems (Schmid-Hempel, 2005). In addition, these studies demonstrated that a specific immune memory that is functionally similar to vertebrate immune memory may be present in invertebrates.

The phenomena of memory-like immune responses are termed immune priming, and examples of pathogen-specific and specific immune priming have been observed in numerous insects. Kurtz and Franz (2003) provided phenomenological evidence for specific memory in the invertebrate immune system by infecting a copepod, *Macrocyclus albidus*, with the tapeworm *Schistocephalus solidus*. Their results showed that the innate defense system of copepods reacted more efficiently during secondary infection after hosts had previously encountered similar parasites. These results demonstrated that the innate immune defense system in invertebrates may have an ability to discriminate amongst antigenic molecules, and that a specific immune memory exists.

In 2006, Sadd and Schmid-Hempel (2006) demonstrated the presence of specific immune priming in the bumblebee (*Bombus terrestris*). They used three different bacterial pathogens to determine whether prior homologous pathogen exposure induces long-term immune protection. As expected, they observed that bees have enhanced protection and specificity during secondary bacterial exposure even if it is several weeks after the initial exposure, suggesting that the invertebrate immune system is capable of specific immune memory. Subsequently, Pham *et al.* (2007) showed that *Streptococcus pneumoniae*-primed flies were protected against a subsequent lethal challenge with *S. pneumoniae*. This response was specific for *S. pneumoniae*, and persisted through the lifetime of the fly. In addition, they suggested that the *Drosophila* Toll pathway, but not the IMD pathway, is required for the primed response.

In 2010, Rodrigues *et al.* (2010) reported that a primed mosquito immune system has a 2- to 3.2-fold enhanced macrophage-like insect cell response upon exposure to a *Plasmodium* malaria parasite, demonstrating that the mosquito immune system can modulate macrophage-like hemocytes. Surprisingly, a strong priming response was also shown when malaria ookinetes breached the mosquito gut barrier and injured epithelial cells upon contact with bacteria, indicating a systemic immune surveillance. Upon re-exposure to a similar insult, mosquitoes mounted a more effective antibacterial response to kill *Plasmodium* parasites. This work is significant in regard to malaria control and the understanding of immune memory in invertebrates. In addition, unraveling the molecular mechanism of insect immune responses, especially the differentiation of mosquito macrophage-like hemocytes, will provide

further insight into the evolution of immune memory in insects.

Three important aspects of invertebrate immune specificity have been surmised (Schulenburg *et al.*, 2007): (1) immune specificity of insects relies on the genetic diversity of pathogen recognition receptors and/or immune effectors; (2) diverse pathogen recognition receptors and/or immune effectors interact synergistically to enhance immune specificity; and (3) specific recognition of pathogens can be greatly enhanced by increasing the concentration of the relevant receptors and/or immune effectors.

Alternative splicing of exons within Dscam immunoglobulin produces diversification, with more than 30,000 isoforms in both *D. melanogaster* and *A. gambiae* (Watson *et al.*, 2005; Dong *et al.*, 2006). Different splice forms show varied affinity with pathogens, as evidence of mammalian immune specificity. However, the molecular mechanism of diversification of Dscams has not yet been determined. In addition, *Drosophila* PGRP-LC, a transmembrane protein, exists as three splice variants (PGRP-LCa, -LCx, and -LCy). LCx functions as a *bona fide* recognition receptor; however, LCa contributes to the transference of immune signaling of monomeric DAP-type PGN for recognition in the IMD pathway. Similarly, LCx homodimers are involved in the polymeric DAP-type PGN recognition pathway, providing yet another piece of evidence that synergistic interaction of pathogen recognition receptors enhances the specificity of pathogen recognition. Finally, many pathogen recognition receptors, such as PGRPs, GNBP, and lectins, are upregulated when hosts are exposed to pathogens, increasing the efficiency of pathogen detection. However, these preliminary observations are quite different from mammalian immune specificity. Further study of immune priming as a consequence of the amount of pathogen is necessary. In addition, because the molecular mechanisms of these observations are entirely unknown, it remains to be demonstrated exactly how the specificity of insect innate immunity is enhanced. In summary, even though immune specificity and immune memory in insect innate immunity are not as developed as adaptive immunity, recent studies suggest that insect innate immunity may be different from that of vertebrates.

14.6.3. Hemimetabolous Insect Immunology

The difference between a holometabolous and hemimetabolous insect is that the former undergoes a pupal stage, whereas the latter does not. Holometabolism, also termed complete metamorphosis, is a development pattern that consists of four life stages: egg, larva, pupa, and adult. Hemimetabolism, termed incomplete metamorphosis, is a developmental process involving three distinct stages: egg, nymph, and adult. The nymph stage is

simply an adult that lacks wings and functional reproductive organs.

Until now, most research regarding insect immunology has been performed using holometabolous insects, such as flies, mosquitoes, silkworms, and beetles. In addition, annotation analysis of insect immune-related genes has been based on information from holometabolous insects, including flies (*Drosophila* spp) (Sackton *et al.*, 2007), mosquitoes (*Aedes aegypti*, *A. gambiae*; Christophides *et al.*, 2002; Waterhouse *et al.*, 2007), bees (*A. mellifera*; Evans *et al.*, 2006), and beetles (*Tribolium castaneum*; Zou *et al.*, 2007). However, genomic information on hemimetabolous insects became available when the International Aphid Genomics Consortium (IAGC) provided results of sequencing of the pea aphid genome (IAGC, 2010). Both the genomes of the pea aphid and its primary symbiont, *Buchnera aphidicola* (Shigenobu *et al.*, 2000), are now available, providing information with regard to the nature of the interdependency between host and symbionts. In addition, this unique genomic resource allows functional genomic studies to better understand the regulatory networks underlying innate immunity and symbiosis.

Recently, Gerardo *et al.* (2010) conducted an annotation analysis of immune- and defense-related genes in the pea aphid genome. They first analyzed the presence or absence of well-known immune gene homologs that have been reported in holometabolous insects. The authors then measured the production of mRNA and protein to gain insight into the pea aphid response to the various pathogenic microbial challenges. We now summarize some interesting results of their study. Several genes related with the Toll pathway, including multiple Toll receptors and Spätzle genes, serine proteases, and serpins, were identified in aphids. However, PGRP genes, well-characterized receptor genes involved in Toll and IMD signaling in holometabolous insects, were not identified, even though two GNBP genes may function as bacterial sensing molecules. Surprisingly, pea aphids do not express many crucial components of the IMD signaling pathway. This pathway is essential for *Drosophila* protection against Gram-negative bacteria. Absence of the IMD pathway-associated genes suggested that pea aphids may express orthologs of the JNK pathway. In *Drosophila*, the JNK pathway is known to regulate developmental processes as well as wound healing. Therefore, the JNK pathway may be essential for the aphid immune response. As described above, the JAK/STAT pathway in holometabolous insects is thought to play an important role in the overproliferation of hemocytes, upregulation of TEP genes, and induction of antiviral responses. Pea aphids express homologs of the core JAK/STAT genes, including genes encoding for the cytokine receptor Domeless, JAK tyrosine kinase, and STAT92E transcription factor. However, an ortholog of the *Drosophila*

JAK/STAT ligand molecule, *upd* (unpaired), was not identified.

As for other immune-related genes, pea aphids do not express many of the AMPs common to other holometabolous insects. For example, whereas all annotated insect genomes have genes that encode defensins, defensin-like AMP genes were not found in pea aphids. In addition, insect Cecropins, Drosocin, Dipterecin, Drosomycin, and Metchnikowin-like AMP genes, which are expressed in *Drosophila*, were not identified. However, surprisingly, six Thaumatin homolog genes, which are well-known AMP genes in plants, were identified. Thaumatin-family proteins contain disulfide-linked polypeptides of approximately 200 residues in length. Recently, some Thaumatin-like proteins were purified from the beetle *T. castaneum*, and were shown to have antifungal activity against filamentous fungi *Beauveria bassiana* and *Fusarium culmorum* (Altincicek *et al.*, 2008). However, why aphids have lost so many common insect AMPs while plant-originated Thaumatin-like proteins are conserved is unknown. Additionally, aphids have five C-type Lectin paralogs and two Galectin paralogs. Insects also express the scavenger receptors, Nimrod and Dscam. In conclusion, pea aphids do not have many genes that function in insect immunology; however, pea aphids have some other genes that may function in the immune response to microbial challenges.

Although symbiosis is outside the scope of this chapter, annotation of the pea aphid and *Buchnera* genomes (Shigenobu *et al.*, 2000) has provided invaluable information on symbiosis. Namely, *Buchnera* has a dramatically small genome, and it was revealed that the *Buchnera* genome has specific genes for the biosynthesis of the nine essential amino acids, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. These amino acids are essential for pea aphid growth; however, aphids do not produce these amino acids. Indeed, the pea aphid genome does not have the genetic machinery required for amino acid synthesis. These data provide invaluable insight into aphid development, symbiotic interactions, and co-evolution with obligate and facultative symbiotic bacteria. In addition, the genomic information of these two species will enable us to characterize novel genes that are involved in the pea aphid immune and defense systems.

14.7. Conclusion

Using multiple approaches, including genetics, cell biology, molecular biology, proteomics, RNAi, and system biology, fruitful research has relied on insect model systems. In addition, the availability of many *Drosophila* mutants that are deficient in immune responses against bacterial and fungal infection has enabled the discovery of several innate immune signaling pathways, such

as Toll, IMD, JNK, and JAK/STAT signaling. However, much has yet to be done regarding the molecular study of insect immunology. For example, the activation mechanism of the microbial proteinase-mediated danger signaling pathway is unclear. How is pro-persephone protease cleaved by microbial proteinases, and what is the protease directly downstream of this signaling pathway? In addition, the structures of ligands that affect these pathways are unknown, whereas the ligand structures that affect the Toll and IMD pathways have been determined. The elucidation of molecular mechanisms and virulence factors involved in the regulation of insect immune pathways will increase our understanding of insect immunology.

In addition, the DAP-type PGN-mediated IMD signal pathway has not been fully elucidated. The identities of the pattern recognition receptors in the *Drosophila* IMD pathway and the recognition mechanism between tracheal cytotoxin (TCT, **Figure 1D**) and PGRP-LE or PGRP-LC have been determined (Chang *et al.*, 2006; Lim *et al.*, 2006). However, TCT is not produced by all Gram-negative bacteria and *bacilli* species. Therefore, a different natural ligand molecule for the IMD pathway from Gram-negative bacteria must exist. Also unknown is how mycoplasma species, which are deficient in PGN in their cell membrane, are recognized by insects, and how the innate immune response after recognition of mycoplasma is activated.

A particular challenge to the insect immunity research community is the determination of the recognition and activation mechanisms after infection with intracellular pathogenic microbes, such as *Listeria monocytogenes* and *Mycobacterium marium*. Recently, Kurata and colleagues discussed the relationship between autophagy and insect innate immunity (Yano *et al.*, 2008; Kurata, 2010). *Drosophila* PGRP-LC and -LE sense the DAP-type PGN of extracellular- and intracellular-infective bacteria and induce several innate immune responses, such as AMP production, via activation of the IMD pathway. Yano *et al.* (2008) observed PGRP-LE-25-dependent autophagy, when *Drosophila* adults were infected with *L. monocytogenes*, which was independent of the IMD pathway. It will be worthwhile to identify the ligand molecules within these intracellular microbes, and to determine the molecular mechanism of DAP-type PGN-dependent autophagy and downstream signaling.

Even though the study of insect antiviral defense mechanisms has made significant progress, we are still far from completely understanding antiviral resistance mechanisms. Insect antiviral mechanisms are quite different from those triggered by bacterial and fungal infections. Future intensive studies regarding the interaction between virus and insect will help unravel antiviral response mechanisms in mammals. In addition, intensive research to understand innate immune responses in diverse insect species is needed.

Our knowledge and understanding of mosquito immunology has greatly advanced during the past 15 years due to the complete genome sequencing of *A. gambiae*. Future studies need to focus on the molecular mechanisms of mosquito immune reactions, and clarify the natural inconsistencies between responses to vectors and parasites. These studies will allow us to develop universal antiparasitic agents, which would be useful for combating malaria. Finally, many viruses, bacteria, protozoans, and metazoans are known to use insects as vectors. Study of *Anopheles* and *Plasmodium* interactions will provide valuable information regarding vector immunity to various human pathogens.

Over the next 10 years, the newly emerging fields reviewed in this chapter, such as gut immunity, immune priming, and hemimetabolous insect immunology, will be studied extensively in diverse research fields. New genomic information from different insect species will help further our understanding of the basic concepts of self and non-self discrimination in the gut, immune priming, immune specificity, and host-symbiont interactions. In particular, determination of the molecular mechanism of symbiosis will be of benefit in understanding mammalian gut immunity.

Finally, basic research of insect immunology will provide a chance to develop new drugs and clinically valuable diagnostic kits. For example, 5-S-GAD (*N*- β -Alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine) was originally isolated as an antibacterial substance from *S. peregrina* adults (Leem *et al.*, 1996). Natori and colleagues recently reported that 5-S-GAD prevents acute lens opacity development due to 5-S-GAD antioxidant activity. Treatment with 5-S-GAD-containing eye drops delayed the progression of UVB-induced cataracts in rats. These studies resulted in lead compounds for drug development, and provide an example of drug discovery based on insect host-defense molecules (Akiyama *et al.*, 2009; Kawada *et al.*, 2009). In addition, because PGN and β -1,3-glucan activates the Toll signaling pathway in insects, which is amplified by a serine protease cascade (Kim *et al.*, 2008), the molecules involved in both PGN recognition and signal amplification are useful for development of novel diagnostic kits. These would be useful for rapid identification of bacteria-contaminated biomaterials, such as platelets and human plasma. A commercially available LPS detection kit has been developed based on the molecular mechanism of the hemolymph clotting system in *Limulus* (Iwanaga *et al.*, 1992). In addition, the components involved in β -1,3-glucan recognition signaling have facilitated the development of sensitive novel kits for the rapid identification of fungi in clinical samples and food products. Recently, a new bacterial contamination detection kit has been developed based on the molecular mechanism of *Tenebrio* Toll signaling pathway.

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References

- Agaisse, H., Petersen, U. M., Boutros, M., Mathey-Prevot, B., & Perrimon, N. (2003). Signaling role of hemocytes in *Drosophila* JAK/STAT-dependent response to septic injury. *Dev. Cell*, 5, 441–450.
- Aggarwal, K., Rus, F., Vriesema-Magnuson, C., Ertürk-Hasdemir, D., Paquette, N., et al. (2008). Rudra interrupts receptor signaling complexes to negatively regulate the IMD pathway. *PLoS Pathog.*, 4, e1000120.
- Akiyama, N., Umeda, I. O., Sogo, S., Nishigori, H., Tsujimoto, M., et al. (2009). 5-S-GAD, a novel radical scavenging compound, prevents lens opacity development. *Free Radic. Biol. Med.*, 46, 511–519.
- Altincicek, B., Knorr, E., & Vilcinskas, A. (2008). Beetle immunity: Identification of immune-inducible genes from the model insect *Tribolium castaneum*. *Dev. Comp. Immunol.*, 32, 585–595.
- An, C., Ishibashi, J., Ragan, E. J., Jiang, H., & Kanost, M. R. (2009). Functions of *Manduca sexta* hemolymph proteinases HP6 and HP8 in two innate immune pathways. *J. Biol. Chem.*, 284, 19716–19726.
- Ashida, M., Doke, K., & Onishi, E. (1974). Activation of prephenoloxidase. 3. Release of a peptide from prephenoloxidase by the activating enzyme. *Biochem. Biophys. Res. Commun.*, 57, 1089–1095.
- Ashida, M., Ishizaki, Y., & Iwahana, H. (1983). Activation of pro-phenoloxidase by bacterial cell walls or beta-1,3-glucans in plasma of the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Commun.*, 113, 562–568.
- Barillas-Mury, C., Charlesworth, A., Gross, I., Richman, A., Hoffmann, J. A., et al. (1996). Immune factor Gambif1, a new rel family member from the human malaria vector, *Anopheles gambiae*. *EMBO J.*, 15, 4691–4701.
- Barondes, S. H. (1984). Soluble lectins: A new class of extracellular proteins. *Science*, 223, 1259–1264.
- Bartel, D. P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell*, 116, 281–297.
- Beck, M. H., & Strand, M. R. (2007). A novel polydnavirus protein inhibits the insect prophenoloxidase activation pathway. *Proc. Natl. Acad. Sci. USA*, 104, 19267–19272.
- Beckage, N. E., & Kanost, M. R. (1993). Effects of parasitism by the braconid wasp *Cotesia congregata* on host hemolymph proteins of the tobacco hornworm, *Manduca sexta*. *Insect Biochem.*, 20, 285–294.
- Belvin, M. P., & Anderson, K. V. (1996). A conserved signaling pathway: The *Drosophila* toll-dorsal pathway. *Annu. Rev. Cell Dev. Biol.*, 12, 393–416.
- Bischoff, V., Vignal, C., Duvic, B., Boneca, I. G., Hoffmann, J. A., et al. (2006). Downregulation of the *Drosophila* immune response by peptidoglycan-recognition proteins SC1 and SC2. *PLoS Pathog.*, 2, e14.
- Boman, H. G., Nilsson, I., & Rasmuson, B. (1972). Inducible anti-bacterial defence system in *Drosophila*. *Nature*, 237, 232–235.

- Broekaert, W. F., Terras, F. R., Cammue, B. P., & Osborn, R. W. (1995). Plant defensins: Novel antimicrobial peptides as components of the host defense system. *Plant Physiol.*, 108, 1353–1358.
- Brown, K. L., & Hancock, R. E. (2006). Cationic host defense (antimicrobial) peptides. *Curr. Opin. Immunol.*, 18, 24–30.
- Buchon, N., Poidevin, M., Kwon, H. M., Guillou, A., Sottas, V., et al. (2009). A single modular serine protease integrates signals from pattern-recognition receptors upstream of the *Drosophila* Toll pathway. *Proc. Natl. Acad. Sci. USA*, 106, 12442–12447.
- Bulet, P. (2003). Antimicrobial peptides in insect immunity. In R. A. Ezekowitz, & J. A. Hoffmann (Eds.). *Innate Immunity. Infectious Diseases* (pp. 89–107). Totowa, NJ: Humana Press.
- Bulet, P., Hetru, C., Dimarcq, J. L., & Hoffmann, D. (1999). Antimicrobial peptides in insects; Structure and function. *Dev. Comp. Immunol.*, 23, 329–344.
- Carlsson, A., Engström, P., Palva, E. T., & Bennich, H. (1991). Attacin, an antibacterial protein from *Hyalophora cecropia*, inhibits synthesis of outer membrane proteins in *Escherichia coli* by interfering with omp gene transcription. *Infect. Immun.*, 59, 3040–3045.
- Casteels, P., Ampe, C., Jacobs, F., Vaeck, M., & Tempst, P. (1989). Antibacterial peptides from honeybees. *EMBO J.*, 8, 2387–2391. Apidaecins: Antimic.
- Cerenius, L., Lee, B. L., & Söderhäll, K. (2008). The proPO-system: Pros and cons for its role in invertebrate immunity. *Trends Immunol.*, 29, 263–271.
- Cerenius, L., Kawabata, S., Lee, B. L., Nonaka, M., & Söderhäll, K. (2010). Proteolytic cascades and their involvement in invertebrate immunity. *Trends Biochem. Sci.*, 35, 575–583.
- Chang, C. I., Chelliah, Y., Borek, D., Mengin-Lecreulx, D., & Deisenhofer, J. (2006). Structure of tracheal cytotoxin in complex with a heterodimeric pattern-recognition receptor. *Science*, 311, 1761–1764.
- Chasan, R., & Anderson, K. V. (1989). The role of easter, an apparent serine protease, in organizing the dorsal-ventral pattern of the *Drosophila* embryo. *Cell*, 56, 391–400.
- Choe, K. M., Werner, T., Stöven, S., Hultmark, D., & Anderson, K. V. (2002). Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in *Drosophila*. *Science*, 296, 359–362.
- Chowdhury, S., Taniai, K., Hara, S., Kadono-Okuda, K., Kato, Y., et al. (1995). cDNA cloning and gene expression of lebecin, a novel member of antibacterial peptides from the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Commun.*, 214, 271–278.
- Christophides, G. K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., et al. (2002). Immunity-related genes and gene families in *Anopheles gambiae*. *Science*, 298, 159–165.
- Cirimotich, C. M., Dong, Y., Garver, L. S., Sim, S., & Dimopoulos, G. (2010). Mosquito immune defenses against *Plasmodium* infection. *Dev. Comp. Immunol.*, 34, 387–395.
- Cociancich, S., Ghazi, A., Hetru, C., Hoffmann, J. A., & Letellier, L. (1993). Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in *Micrococcus luteus*. *J. Biol. Chem.*, 268, 19239–19245.
- Collins, F. H., Sakai, R. K., Vernick, K. D., Paskewitz, S., Seeley, D. C., et al. (1986). Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. *Science*, 234, 607–610.
- Crozatier, M., & Meister, M. (2007). *Drosophila* haematopoiesis. *Cell Microbiol.*, 9, 1117–1126.
- Cudic, M., Bulet, P., Hoffmann, R., Craik, D. J., & Otvos, L., Jr. (1999). Chemical synthesis, antibacterial activity and conformation of dipterecin, an 82-mer peptide originally isolated from insects. *Eur. J. Biochem.*, 266, 549–558.
- Daquinag, A. C., Nakamura, S., Takao, T., Shimonishi, Y., & Tsukamoto, T. (1995). Primary structure of a potent endogenous dopa-containing inhibitor of phenol oxidase from *Musca domestica*. *Proc. Natl. Acad. Sci. USA*, 92, 2964–2968.
- Dearolf, C. R., Topol, J., & Parker, C. S. (1989). The caudal gene product is a direct activator of fushi tarazu transcription during *Drosophila* embryogenesis. *Nature*, 341, 340–343.
- Deddouche, S., Matt, N., Budd, A., Mueller, S., Kemp, C., et al. (2008). The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in *Drosophila*. *Nat. Immunol.*, 9, 1425–1432.
- De Gregorio, E., Han, S. J., Lee, W. J., Baek, M. J., Osaki, T., et al. (2002). An immune-responsive Serpin regulates the melanization cascade in *Drosophila*. *Dev. Cell*, 3, 581–592.
- Dimopoulos, G., Richman, A., Müller, H. M., & Kafatos, F. C. (1997). Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proc. Natl. Acad. Sci. USA*, 94, 11508–11513.
- Dimopoulos, G., Seeley, D., Wolf, A., & Kafatos, F. C. (1998). Malaria infection of the mosquito *Anopheles gambiae* activates immune-responsive genes during critical transition stages of the parasite life cycle. *EMBO J.*, 17, 6115–6123.
- Dimopoulos, G., Müller, H. M., Levashina, E. A., & Kafatos, F. C. (2001). Innate immune defense against malaria infection in the mosquito. *Curr. Opin. Immunol.*, 13, 79–88.
- Dohke, K. (1973). Studies on prephenoloxidase-activating enzyme from cuticle of the silkworm *Bombyx mori*. II. Purification and characterization of the enzyme. *Arch. Biochem. Biophys.*, 157, 210–221.
- Dong, Y., Taylor, H. E., Dimopoulos, G., & AgDscam (2006). AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system. *PLoS Biol.*, 4, e229.
- Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., et al. (2005). The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *Drosophila*. *Nat. Immunol.*, 6, 946–953.
- Eleftherianos, I., Millichap, P. J., French-Constant, R. H., & Reynolds, S. E. (2006). RNAi suppression of recognition protein mediated immune responses in the tobacco hornworm *Manduca sexta* causes increased susceptibility to the insect pathogen *Photobacterium*. *Dev. Comp. Immunol.*, 30, 1099–1107.
- Engström, A., Engström, P., Tao, Z. J., Carlsson, A., & Bennich, H. (1984). Insect immunity. The primary structure of the antibacterial protein attacin F and its relation to two native attacins from *Hyalophora cecropia*. *EMBO J.*, 3, 2065–2070.

- Engström, Y., Kadalayil, L., Sun, S. C., Samakovlis, C., & Hultmark, D. (1993). Kappa B-like motifs regulate the induction of immune genes in *Drosophila*. *J. Mol. Biol.*, 232, 327–333.
- Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J. L., et al. (2006). Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Mol. Biol.*, 15, 645–656.
- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., et al. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391, 806–811.
- Fradin, C., Poulain, D., & Jouault, T. (2000). Beta-1,2-linked oligomannosides from *Candida albicans* bind to a 32-kilodalton macrophage membrane protein homologous to the mammalian lectin galectin-3. *Infect. Immun.*, 68, 4391–4398.
- Franc, N. C., Heitzler, P., Ezekowitz, R. A., & White, K. (1999). Requirement for croquemort in phagocytosis of apoptotic cells in *Drosophila*. *Science*, 284, 1991–1994.
- Frolet, C., Thoma, M., Blandin, S., Hoffmann, J. A., & Leвшина, E. A. (2006). Boosting NF-kappaB-dependent basal immunity of *Anopheles gambiae* aborts development of *Plasmodium berghei*. *Immunity*, 25, 677–685.
- Fujimoto, K., Okino, N., Kawabata, S., Iwanaga, S., & Ohnishi, E. (1995). Nucleotide sequence of the cDNA encoding the proenzyme of phenol oxidase A1 of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA*, 92, 7769–7773.
- Galiana-Arnoux, D., Dostert, C., Schneemann, A., Hoffmann, J. A., & Imler, J. L. (2006). Essential function *in vivo* for Dicer-2 in host defense against RNA viruses in *Drosophila*. *Nat. Immunol.*, 7, 590–597.
- Garver, L. S., Dong, Y., & Dimopoulos, G. (2009). Caspar controls resistance to *Plasmodium falciparum* in diverse anopheline species. *PLoS Pathog.*, 5, e1000335.
- Georgel, P., Naitza, S., Kappler, C., Ferrandon, D., Zachary, D., et al. (2001). *Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis. *Dev. Cell*, 1, 503–514.
- Gerardo, N. M., Altincicek, B., Anselme, C., Atamian, H., Barribeau, S. M., et al. (2010). Immunity and other defenses in pea aphids, *Acyrtosiphon pisum*. *Genome Biol.*, 11, R21.
- Glaser, R. W. (1918). On the existence of immunity principles in insects. *Psyche*, 25, 38–46.
- Gobert, V., Gottar, M., Matskevich, A. A., Rutschmann, S., Royet, J., et al. (2003). Dual activation of the *Drosophila* toll pathway by two pattern recognition receptors. *Science*, 302, 2126–2130.
- Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., et al. (2002). The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. *Nature*, 416, 640–644.
- Gottar, M., Gobert, V., Matskevich, A. A., Reichhart, J. M., Wang, C., et al. (2006). Dual detection of fungal infections in *Drosophila* via recognition of glucans and sensing of virulence factors. *Cell*, 127, 1425–1437.
- Ha, E. M., Oh, C. T., Bae, Y. S., & Lee, W. J. (2005a). A direct role for dual oxidase in *Drosophila* gut immunity. *Science*, 310, 847–850.
- Ha, E. M., Oh, C. T., Ryu, J. H., Bae, Y. S., Kang, S. W., et al. (2005b). An antioxidant system required for host protection against gut infection in *Drosophila*. *Dev. Cell*, 8, 125–132.
- Ha, E. M., Lee, K. A., Park, S. H., Kim, S. H., Nam, H. J., et al. (2009a). Regulation of DUOX by the galphaprophospholipase C beta-Ca²⁺ pathway in *Drosophila* gut immunity. *Dev. Cell*, 16, 386–397.
- Ha, E. M., Lee, K. A., Seo, Y. Y., Kim, S. H., Lim, J. H., et al. (2009b). Coordination of multiple dual oxidase regulatory pathways in responses to commensal and infectious microbes in *Drosophila* gut. *Nat. Immunol.*, 10, 949–957.
- Hall, M., Scott, T., Sugumaran, M., Söderhäll, K., Law, J. H., et al. (1995). Proenzyme of *Manduca sexta* phenol oxidase: Purification, activation, substrate specificity of the active enzyme, and molecular cloning. *Proc. Natl. Acad. Sci. USA*, 92, 7764–7768.
- Hammond, S. M. (2005). Dicing and slicing: The core machinery of the RNA interference pathway. *FEBS Lett.*, 579, 5822–5829.
- Hashimoto, C., Kim, D. R., Weiss, L. A., Miller, J. W., Morisato, D., et al. (2003). Spatial regulation of developmental signaling by a serpin. *Dev. Cell*, 5, 945–950.
- Hashimoto, Y., Tabuchi, Y., Sakurai, K., Kutsuna, M., Kurokawa, K., et al. (2009). Identification of lipoteichoic acid as a ligand for draper in the phagocytosis of *Staphylococcus aureus* by *Drosophila* hemocytes. *J. Immunol.*, 183, 7451–7460.
- Hennessy, E. J., Parker, A. E., & O'Neill, L. A. (2010). Targeting Toll-like receptors: Emerging therapeutics? *Nat. Rev. Drug Discov.*, 9, 293–307.
- Hetru, C., & Hoffmann, J. A. (2009). NF-kappaB in the immune response of *Drosophila*. *Cold Spring Harb. Perspect. Biol.*, 1, a000232.
- IAGC (International Aphid genomics Consortium) (2010). Genome sequence of the pea aphid *Acyrtosiphon pisum*. *PLoS Biol.*, 8, e1000313.
- Iijima, R., Kurata, S., & Natori, S. (1993). Purification, characterization, and cDNA cloning of an antifungal protein from the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae. *J. Biol. Chem.*, 268, 12055–12061.
- Imler, J. L., & Bulet, P. (2005). Antimicrobial peptides in *Drosophila*: Structures, activities and gene regulation. *Chem. Immunol. Allergy*, 86, 1–21.
- Iwanaga, S., Miyata, T., Tokunaga, F., & Muta, T. (1992). Molecular mechanism of hemolymph clotting system in *Limulus*. *Thromb. Res.*, 68, 1–32.
- Janeway, C. A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.*, 54(Pt. 1), 1–13.
- Jang, I. H., Chosa, N., Kim, S. H., Nam, H. J., Lemaitre, B., et al. (2006). A Spätzle-processing enzyme required for toll signaling activation in *Drosophila* innate immunity. *Dev. Cell*, 10, 45–55.
- Jiang, H., & Kanost, M. R. (1997). Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from *Manduca sexta*. *J. Biol. Chem.*, 272, 1082–1087.
- Jiang, H., & Kanost, M. R. (2000). The clip-domain family of serine proteinases in arthropods. *Insect Biochem. Mol. Biol.*, 30, 95–105.
- Jiang, H., Wang, Y., & Kanost, M. R. (1998). Pro-phenol oxidase activating proteinase from an insect, *Manduca sexta*: A bacteria-inducible protein similar to *Drosophila* easter. *Proc. Natl. Acad. Sci. USA*, 95, 12220–12225.

- Jiang, R., Kim, E. H., Gong, J. H., Kwon, H. M., Kim, C. H., et al. (2009). Three pairs of protease-serpin complexes cooperatively regulate the insect innate immune responses. *J. Biol. Chem.*, 284, 35652–35658.
- Ju, J. S., Cho, M. H., Brade, L., Kim, J. H., Park, J. W., et al. (2006). A novel 40-kDa protein containing six repeats of an epidermal growth factor-like domain functions as a pattern recognition protein for lipopolysaccharide. *J. Immunol.*, 177, 1838–1845.
- Jutras, I., & Desjardins, M. (2005). Phagocytosis: At the crossroads of innate and adaptive immunity. *Annu. Rev. Cell Dev. Biol.*, 21, 511–527.
- Kambris, Z., Brun, S., Jang, I. H., Nam, H. J., Romeo, Y., et al. (2006). *Drosophila* immunity: A large-scale *in vivo* RNAi screen identifies five serine proteases required for Toll activation. *Curr. Biol.*, 16, 808–813.
- Kan, H., Kim, C. H., Kwon, H. M., Park, J. W., Roh, K. B., et al. (2008). Molecular control of phenoloxidase-induced melanin synthesis in an insect. *J. Biol. Chem.*, 283, 25316–25323.
- Kang, D., Liu, G., Lundström, A., Gelius, E., & Steiner, H. (1998). A peptidoglycan recognition protein in innate immunity conserved from insects to humans. *Proc. Natl. Acad. Sci. USA*, 95, 10078–10082.
- Kanost, M. R., & Jiang, H. (1997). Serpins from an insect, *Manduca sexta*. *Adv. Exp. Med. Biol.*, 425, 155–161.
- Kanost, M. R., Jiang, H., & Yu, X. Q. (2004). Innate immune responses of a lepidopteran insect, *Manduca sexta*. *Immunol. Rev.*, 198, 97–105.
- Kawabata, T., Yasuhara, Y., Ochiai, M., Matsuura, S., & Ashida, M. (1995). Molecular cloning of insect pro-phenol oxidase: A copper-containing protein homologous to arthropod hemocyanin. *Proc. Natl. Acad. Sci. USA*, 92, 7774–7778.
- Kawada, H., Kojima, M., Kimura, T., Natori, S., Sasaki, K., et al. (2009). Effect of 5-S-GAD on UV-B-induced cataracts in rats. *Jpn J. Ophthalmol.*, 53, 531–535.
- Kawaguchi, N., Komano, H., & Natori, S. (1991). Involvement of *Sarcophaga* lectin in the development of imaginal discs of *Sarcophaga peregrina* in an autocrine manner. *Dev. Biol.*, 144, 86–93.
- Kawai, T., & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors. *Nat. Immunol.*, 11, 373–384.
- Kemp, C., & Imler, J. L. (2009). Antiviral immunity in *Drosophila*. *Curr. Opin. Immunol.*, 21, 3–9.
- Kim, C. H., Kim, S. J., Kan, H., Kwon, H. M., Roh, K. B., et al. (2008). A three-step proteolytic cascade mediates the activation of the peptidoglycan-induced Toll pathway in an insect. *J. Biol. Chem.*, 283, 7599–7607.
- Kim, M., Lee, J. H., Lee, S. Y., Kim, E., & Chung, J. (2006). Caspar, a suppressor of antibacterial immunity in *Drosophila*. *Proc. Natl. Acad. Sci. USA*, 103, 16358–16363.
- Kim, M. S., Baek, M. J., Lee, M. H., Park, J. W., Lee, S. Y., et al. (2002). A new easter-type serine protease cleaves a masquerade-like protein during prophenoloxidase activation in *Holotrichia diomphalia* larvae. *J. Biol. Chem.*, 277, 39999–40004.
- Kleino, A., Myllymäki, H., Kallio, J., Vanha-aho, L. M., Oksanen, K., et al. (2008). Pirk is a negative regulator of the *Drosophila* Imd pathway. *J. Immunol.*, 180, 5413–5422.
- Kocks, C., Cho, J. H., Nehme, N., Ulvila, J., Pearson, A. M., et al. (2005). Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in *Drosophila*. *Cell*, 123, 335–346.
- Koizumi, N., Imai, Y., Morozumi, A., Imamura, M., Kadotani, T., et al. (1999). Lipopolysaccharide-binding protein of *Bombyx mori* participates in a hemocyte-mediated defense reaction against gram-negative bacteria. *J. Insect. Physiol.*, 45, 853–859.
- Komano, H., Nozawa, R., Mizuno, D., & Natori, S. (1983). Measurement of *Sarcophaga peregrina* lectin under various physiological conditions by radioimmunoassay. *J. Biol. Chem.*, 258, 2143–2147.
- Krem, M. M., & Di Cera, E. (2002). Evolution of enzyme cascades from embryonic development to blood coagulation. *Trends Biochem. Sci.*, 27, 67–74.
- Krzemień, J., Dubois, L., Makki, R., Meister, M., Vincent, A., et al. (2007). Control of blood cell homeostasis in *Drosophila* larvae by the posterior signalling centre. *Nature*, 446, 325–328.
- Kuraishi, T., Manaka, J., Kono, M., Ishii, H., Yamamoto, N., et al. (2007). Identification of calreticulin as a marker for phagocytosis of apoptotic cells in *Drosophila*. *Exp. Cell Res.*, 313, 500–510.
- Kuraishi, T., Manaka, J., Kono, M., Ishii, H., Yamamoto, N., et al. (2009). Pretaporter, a *Drosophila* protein serving as a ligand for Draper in the phagocytosis of apoptotic cells. *EMBO J.*, 28, 3868–3878.
- Kurata, S. (2010). Extracellular and intracellular pathogen recognition by *Drosophila* PGRP-LE and PGRP-LC. *Intl. Immunol.*, 22, 143–148.
- Kurtz, J., & Franz, K. (2003). Innate defence: Evidence for memory in invertebrate immunity. *Nature*, 425, 37–38.
- Kurucz, E., Márkus, R., Zsámboki, J., Folkl-Medzihradsky, K., Darula, Z., et al. (2007). Nimrod, a putative phagocytosis receptor with EGF repeats in *Drosophila* plasmatocytes. *Curr. Biol.*, 17, 649–654.
- Kwon, T. H., Kim, M. S., Choi, H. W., Joo, C. H., Cho, M. Y., et al. (2000). A masquerade-like serine proteinase homologue is necessary for phenoloxidase activity in the coleopteran insect, *Holotrichia diomphalia* larvae. *Eur. J. Biochem.*, 267, 6188–6196.
- Levashina, E. A., Langley, E., Green, C., Gubb, D., Ashburner, M., Hoffmann, J. A., & Reichhart, J. M. (1999). Constitutive activation of toll-mediated antifungal defense in serpin-deficient *Drosophila*. *Science*, 285, 1917–1919.
- Lee, S. Y., Moon, H. J., Kurata, S., Kurama, T., Natori, S., et al. (1994). Purification and molecular cloning of cDNA for an inducible antibacterial protein of larvae of a coleopteran insect, *Holotrichia diomphalia*. *J. Biochem.*, 115, 82–86.
- Lee, S. Y., Moon, H. J., Kurata, S., Natori, S., & Lee, B. L. (1995). Purification and cDNA cloning of an antifungal protein from the hemolymph of *Holotrichia diomphalia* larvae. *Biol. Pharm. Bull.*, 18, 1049–1052.
- Lee, S. Y., Cho, M. Y., Hyun, J. H., Lee, K. M., Homma, K. I., et al. (1998). Molecular cloning of cDNA for prophenoloxidase-activating factor I, a serine protease is induced by lipopolysaccharide or 1,3-beta-glucan in coleopteran insect, *Holotrichia diomphalia* larvae. *Eur. J. Biochem*, 257, 615–621.

- Lee, W. J., Lee, J. D., Kravchenko, V. V., Ulevitch, R. J., & Brey, P. T. (1996). Purification and molecular cloning of an inducible gram-negative bacteria-binding protein from the silkworm, *Bombyx mori*. *Proc. Natl. Acad. Sci. USA*, 93, 7888–7893.
- Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., et al. (2004). Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*, 117, 69–81.
- Leem, J. Y., Nishimura, C., Kurata, S., Shimada, I., Kobayashi, A., et al. (1996). Purification and characterization of N-beta-alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine, a novel antibacterial substance of *Sarcophaga peregrina* (flesh fly). *J. Biol. Chem.*, 271, 13573–13577.
- Lemaitre, B., & Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.*, 25, 697–743.
- Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., & Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell*, 86, 973–983.
- Leto, T. L., & Geiszt, M. (2006). Role of Nox family NADPH oxidases in host defense. *Antioxid. Redox Signal*, 8, 1549–1561.
- Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J. H., Caroff, M., et al. (2003). The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. *Nat. Immunol.*, 4, 478–484.
- Levashina, E. A., Langley, E., Green, C., Gub, D., Ashburner, M., et al. (1999). Constitutive activation of toll-mediated antifungal defense in serpin-deficient *Drosophila*. *Science*, 285, 1917–1919.
- Levashina, E. A., Moita, L. F., Blandin, S., Vriend, G., Lagueux, M., et al. (2001). Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. *Cell*, 104, 709–718.
- Lhocine, N., Ribeiro, P. S., Buchon, N., Wepf, A., Wilson, R., et al. (2008). PIMS modulates immune tolerance by negatively regulating *Drosophila* innate immune signaling. *Cell Host Microbe*, 4, 147–158.
- Li, W. X., & Ding, S. W. (2001). Viral suppressors of RNA silencing. *Curr. Opin. Biotechnol.*, 12, 150–154.
- Li, Y., Wang, Y., Jiang, H., & Deng, J. (2009). Crystal structure of *Manduca sexta* prophenoloxidase provides insights into the mechanism of type 3 copper enzymes. *Proc. Natl. Acad. Sci. USA*, 106, 17002–17006.
- Ligoxygakis, P., Pelte, N., Hoffmann, J. A., & Reichhart, J. M. (2002a). Activation of *Drosophila* Toll during fungal infection by a blood serine protease. *Science*, 297, 114–116.
- Ligoxygakis, P., Pelte, N., Ji, C., Leclerc, V., Duvic, B., et al. (2002b). A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*. *EMBO J.*, 21, 6330–6337.
- Ligoxygakis, P., Roth, S., & Reichhart, J. M. (2003). A serpin regulates dorsal-ventral axis formation in the *Drosophila* embryo. *Curr. Biol.*, 13, 2097–2102.
- Lim, J. H., Kim, M. S., Kim, H. E., Yano, T., Oshima, Y., et al. (2006). Structural basis for preferential recognition of diaminopimelic acid-type peptidoglycan by a subset of peptidoglycan recognition proteins. *J. Biol. Chem.*, 281, 8286–8295.
- Lu, Z., & Jiang, H. (2007). Regulation of phenoloxidase activity by high- and low-molecular-weight inhibitors from the larval hemolymph of *Manduca sexta*. *Insect Biochem. Mol. Biol.*, 37, 478–485.
- Mackenzie, J. S., Gubler, D. J., & Petersen, L. R. (2004). Emerging flaviviruses: The spread and resurgence of Japanese encephalitis, West Nile and Dengue viruses. *Nat. Med.*, 10, 98–109.
- Maillet, F., Bischoff, V., Vignal, C., Hoffmann, J., & Royet, J. (2008). The *Drosophila* peptidoglycan recognition protein PGRP-LF blocks PGRP-LC and IMD/JNK pathway activation. *Cell Host Microbe*, 3, 293–303.
- Makki, R., Meister, M., Pennetier, D., Ubeda, J. M., Braun, A., et al. (2010). A short receptor downregulates Jak/STAT signalling to control the *Drosophila* cellular immune response. *PLoS Biol.*, 8, e1000441.
- Manaka, J., Kuraishi, T., Shiratsuchi, A., Nakai, Y., Higashida, H., et al. (2004). Draper-mediated and phosphatidylserine-independent phagocytosis of apoptotic cells by *Drosophila* hemocytes/macrophages. *J. Biol. Chem.*, 279, 48466–48476.
- Mason, H. S. (1955). Comparative biochemistry of the phenoloxidase complex. *Adv. Enzymol.*, 16, 105–184.
- Matsushita, M., & Fujita, T. (1992). Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. *J. Exp. Med.*, 176, 1497–1502.
- Medzhitov, R., & Janeway, C. A. (2002). Decoding the patterns of self and nonself by the innate immune system. *Science*, 296, 298–300.
- Medzhitov, R., Preston-Hurlburt, P., & Janeway, C. A. (1997). A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature*, 388, 394–397.
- Meister, C.M., & Lagueux, M. (2003). *Drosophila* blood cells. *Cell Microbiol.*, 5, 573–580.
- Michel, K., Budd, A., Pinto, S., Gibson, T. J., & Kafatos, F. C. (2005). *Anopheles gambiae* SRPN2 facilitates midgut invasion by the malaria parasite *Plasmodium berghei*. *EMBO Rep.*, 6, 891–897.
- Michel, T., Reichhart, J. M., Hoffmann, J. A., & Royet, J. (2001). *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature*, 414, 756–759.
- Mishima, Y., Quintin, J., Aimaniananda, V., Kellenberger, C., Coste, F., et al. (2009). The N-terminal domain of *Drosophila* Gram-negative binding protein 3 (GNBP3) defines a novel family of fungal pattern recognition receptors. *J. Biol. Chem.*, 284, 28687–28697.
- Nakajima, H., Komano, H., Esumi-Kurusu, M., Abe, S., Yamazaki, M., et al. (1982). Induction of macrophage-mediated tumor lysis by an animal lectin, *Sarcophaga peregrina* agglutinin. *Gann.*, 73, 627–632.
- Nappi, A. J., Frey, F., & Carton, Y. (2005). *Drosophila* serpin 27A is a likely target for immune suppression of the blood cell-mediated melanotic encapsulation response. *J. Insect Physiol.*, 51, 197–205.
- Natori, S., Shiraishi, H., Hori, S., & Kobayashi, A. (1999). The roles of *Sarcophaga* defense molecules in immunity and metamorphosis. *Dev. Comp. Immunol.*, 23, 317–328.

- Ochiai, M., & Ashida, M. (2000). A pattern-recognition protein for beta-1,3-glucan. The binding domain and the cDNA cloning of beta-1,3-glucan recognition protein from the silkworm, *Bombyx mori*. *J. Biol. Chem.*, 275, 4995–5002.
- Ohnishi, M. (1953). Tyrosinase activity during puparium formation in *Drosophila melanogaster*. *Jpn. J. Zool.*, 11, 69–74.
- Okada, M., & Natori, S. (1983). Purification and characterization of an antibacterial protein from haemolymph of *Sarcophaga peregrina* (flesh-fly) larvae. *Biochem. J.*, 211, 727–734.
- Osta, M. A., Christophides, G. K., & Kafatos, F. C. (2004). Effects of mosquito genes on *Plasmodium* development. *Science*, 303, 2030–2032.
- Pace, K. E., Lebestky, T., Hummel, T., Arnoux, P., Kwan, K., et al. (2002). Characterization of a novel *Drosophila melanogaster* galectin. Expression in developing immune, neural, and muscle tissues. *J. Biol. Chem.*, 277, 13091–13098.
- Padovan, L., Scocchi, M., & Tossi, A. (2010). Structural aspects of plant antimicrobial peptides. *Curr. Protein Pept. Sci.*, 11, 210–219.
- Park, J. W., Kim, C. H., Kim, J. H., Je, B. R., Roh, K. B., et al. (2007). Clustering of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects. *Proc. Natl. Acad. Sci. USA*, 104, 6602–6607.
- Pham, L. N., Dionne, M. S., Shirasu-Hiza, M., & Schneider, D. S. (2007). A specific primed immune response in *Drosophila* is dependent on phagocytes. *PLoS Pathog.*, 3, e26.
- Philips, J. A., Rubin, E. J., & Perrimon, N. (2005). *Drosophila* RNAi screen reveals CD36 family member required for mycobacterial infection. *Science*, 309, 1251–1253.
- Piao, S., Song, Y. L., Kim, J. H., Park, S. Y., Park, J. W., et al. (2005). Crystal structure of a clip-domain serine protease and functional roles of the clip domains. *EMBO J.*, 24, 4404–4414.
- Piao, S., Kim, S., Kim, J. H., Park, J. W., Lee, B. L., et al. (2007). Crystal structure of the serine protease domain of prophenoloxidase activating factor-I. *J. Biol. Chem.*, 282, 10783–10791.
- Povelones, M., Waterhouse, R. M., Kafatos, F. C., & Christophides, G. K. (2009). Leucine-rich repeat protein complex activates mosquito complement in defense against *Plasmodium parasites*. *Science*, 324, 258–261.
- Pye, A. E. (1974). Microbial activation of prophenoloxidase from immune insect larvae. *Nature*, 251, 610–613.
- Rabinovich, G. A., Baum, L. G., Tinari, N., Paganelli, R., Natoli, C., et al. (2002). Galectins and their ligands: Amplifiers, silencers or tuners of the inflammatory response?. *Trends Immunol.*, 23, 313–320.
- Ragan, E. J., An, C., Yang, C. T., & Kanost, M. R. (2010). Analysis of mutually exclusive alternatively spliced serpin-1 isoforms and identification of serpin-1 proteinase complexes in *Manduca sexta* hemolymph. *J. Biol. Chem.*, 285, 29642–29650.
- Rahnamaeian, M., Langen, G., Imani, J., Khalifa, W., Altincicek, B., et al. (2009). Insect peptide metchnikowin confers on barley a selective capacity for resistance to fungal ascomycetes pathogens. *J. Exp. Bot.*, 60, 4105–4114.
- Rämet, M., Pearson, A., Manfrulli, P., Li, X., Koziel, H., et al. (2001). *Drosophila* scavenger receptor CI is a pattern recognition receptor for bacteria. *Immunity*, 15, 1027–1038.
- Rämet, M., Manfrulli, P., Pearson, A., Mathey-Prevot, B., & Ezekowitz, R. A. (2002). Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for *E. coli*. *Nature*, 416, 644–648.
- Rees, J. A., Moniatte, M., & Bulet, P. (1997). Novel antibacterial peptides isolated from a European bumblebee, *Bombus pascuorum* (Hymenoptera, Apoidea). *Insect Biochem. Mol. Biol.*, 27, 413–422.
- Reichhart, J. M. (2005). Tip of another iceberg: *Drosophila* serpins. *Trends Cell Biol.*, 15, 659–665.
- Rizki, R. M., & Rizki, T. M. (1984). Selective destruction of a host blood cell type by a parasitoid wasp. *Proc. Natl. Acad. Sci. USA*, 81, 6154–6158.
- Rizki, T., & Rizki, R. (1990). Encapsulation of parasitoid eggs in phenoloxidase-deficient mutants of *Drosophila melanogaster*. *J. Insect. Physiol.*, 36, 523–529.
- Rodrigues, J., Brayner, F. A., Alves, L. C., Dixit, R., & Barillas-Mury, C. (2010). Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. *Science*, 329, 1353–1355.
- Roh, K. B., Kim, C. H., Lee, H., Kwon, H. M., Park, J. W., et al. (2009). Proteolytic cascade for the activation of the insect toll pathway induced by the fungal cell wall component. *J. Biol. Chem.*, 284, 19474–19481.
- Royet, J., & Dziarski, R. (2007). Peptidoglycan recognition proteins: Pleiotropic sensors and effectors of antimicrobial defences. *Nat. Rev. Microbiol.*, 5, 264–277.
- Ryu, J. H., Kim, S. H., Lee, H. Y., Bai, J. Y., Nam, Y. D., et al. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. *Science*, 319, 777–782.
- Sabatier, L., Jouanguy, E., Dostert, C., Zachary, D., Dimarçq, J. L., et al. (2003). Pherokine-2 and -3. *Eur. J. Biochem.*, 270, 3398–3407.
- Sabin, L. R., Hanna, S. L., & Cherry, S. (2010). Innate antiviral immunity in *Drosophila*. *Curr. Opin. Immunol.*, 22, 4–9.
- Sackton, T. B., Lazzaro, B. P., Schlenke, T. A., Evans, J. D., Hultmark, D., et al. (2007). Dynamic evolution of the innate immune system in *Drosophila*. *Nat. Genet.*, 39, 1461–1468.
- Sadd, B. M., & Schmid-Hempel, P. (2006). Insect immunity shows specificity in protection upon secondary pathogen exposure. *Curr. Biol.*, 16, 1206–1210.
- Scherfer, C., Tang, H., Kambris, Z., Lhocine, N., Hashimoto, C., et al. (2008). *Drosophila* Serpin-28D regulates hemolymph phenoloxidase activity and adult pigmentation. *Dev. Biol.*, 323, 189–196.
- Schleifer, K. H., & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.*, 36, 407–477.
- Schmid-Hempel, P. (2005). Natural insect host-parasite systems show immune priming and specificity: Puzzles to be solved. *Bioessays*, 27, 1026–1034.
- Schulenburg, H., Boehnisch, C., & Michiels, N. K. (2007). How do invertebrates generate a highly specific innate immune response? *Mol. Immunol.*, 44, 3338–3344.
- Selsted, M. E., Harwig, S. S., Ganz, T., Schilling, J. W., & Lehrer, R. I. (1985). Primary structures of three human neutrophil defensins. *J. Clin. Invest.*, 76, 1436–1439.

- Shelby, K. S., Adeyeye, O. A., Okot-Kotber, B. M., & Webb, B. A. (2000). Parasitism-linked block of host plasma melanization. *J. Invertebr. Pathol.*, 75, 218–225.
- Shi, L., Li, B., & Paskewitz, S. M. (2006). Cloning and characterization of a putative inhibitor of melanization from *Anopheles gambiae*. *Insect Mol. Biol.*, 15, 313–320.
- Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., & Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature*, 407, 81–86.
- Silverman, G. A., Whisstock, J. C., Bottomley, S. P., Huntington, J. A., Kaiserman, D., et al. (2010). Serpins flex their muscle: I. Putting the clamps on proteolysis in diverse biological systems. *J. Biol. Chem.*, 285, 24299–24305.
- Sinden, R. E. (2002). Molecular interactions between *Plasmodium* and its insect vectors. *Cell Microbiol.*, 4, 713–724.
- Sinden, R. E. (2004). A proteomic analysis of malaria biology: Integration of old literature and new technologies. *Intl. J. Parasitol.*, 34, 1441–1450.
- Sommer, M. O., Dantas, G., & Church, G. M. (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science*, 325, 1128–1131.
- Sorrentino, R. P., Melk, J. P., & Govind, S. (2004). Genetic analysis of contributions of dorsal group and JAK-Stat92E pathway genes to larval hemocyte concentration and the egg encapsulation response in *Drosophila*. *Genetics*, 166, 1343–1356.
- Souza-Neto, J. A., Sim, S., & Dimopoulos, G. (2009). An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Proc. Natl. Acad. Sci. USA*, 106, 17841–17846.
- Sroka, P., & Vinson, S. B. (1978). Phenoloxidase activity in the hemolymph of parasitized and unparasitized *Heliothis virescens*. *Insect Biochem.*, 8, 399–402.
- Steiner, H., Hultmark, D., Engström, A., Bennich, H., & Boman, H. G. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature*, 292, 246–248.
- Stevens, J. M. (1962). Bactericidal activity of the blood of actively immunized wax moth larvae. *Can. J. Microbiol.*, 8, 491–499.
- Stuart, L. M., & Ezekowitz, R. A. (2008). Phagocytosis and comparative innate immunity: Learning on the fly. *Nat. Rev. Immunol.*, 8, 131–141.
- Stuart, L. M., Boulais, J., Charriere, G. M., Hennessy, E. J., Brunet, S., et al. (2007). A systems biology analysis of the *Drosophila* phagosome. *Nature*, 445, 95–101.
- Su, X. D., Gastinel, L. N., Vaughn, D. E., Faye, I., Poon, P., et al. (1998). Crystal structure of hemolin: A horseshoe shape with implications for homophilic adhesion. *Science*, 281, 991–995.
- Sun, S. C., Lindström, I., Boman, H. G., Faye, I., & Schmidt, O. (1990). Hemolin: An insect-immune protein belonging to the immunoglobulin superfamily. *Science*, 250, 1729–1732.
- Takehana, A., Katsuyama, T., Yano, T., Oshima, Y., Takada, H., et al. (2002). Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in *Drosophila* larvae. *Proc. Natl. Acad. Sci. USA*, 99, 13705–13710.
- Takeuchi, O., & Akira, S. (2008). MDA5/RIG-I and virus recognition. *Curr. Opin. Immunol.*, 20, 17–22.
- Tang, H., Kambris, Z., Lemaitre, B., & Hashimoto, C. (2008). A serpin that regulates immune melanization in the respiratory system of *Drosophila*. *Dev. Cell.*, 15, 617–626.
- Vance, V., & Vaucheret, H. (2001). RNA silencing in plants – defense and counterdefense. *Science*, 292, 2277–2280.
- Volz, J., Müller, H. M., Zdanowicz, A., Kafatos, F. C., & Osta, M. A. (2006). A genetic module regulates the melanization response of *Anopheles* to *Plasmodium*. *Cell Microbiol.*, 8, 1392–1405.
- Wang, X. H., Aliyari, R., Li, W. X., Li, H. W., Kim, K., et al. (2006). RNA interference directs innate immunity against viruses in adult *Drosophila*. *Science*, 312, 452–454.
- Waterhouse, R. M., Kriventseva, E. V., Meister, S., Xi, Z., Alvarez, K. S., et al. (2007). Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. *Science*, 316, 1738–1743.
- Watson, F. L., Püttmann-Holgado, R., Thomas, F., Lamar, D. L., Hughes, M., et al. (2005). Extensive diversity of Ig33 superfamily proteins in the immune system of insects. *Science*, 309, 1874–1878.
- Weber, A. N., Tauszig-Delamasure, S., Hoffmann, J. A., Lelièvre, E., Gascan, H., et al. (2003). Binding of the *Drosophila* cytokine Spätzle to Toll is direct and establishes signaling. *Nat. Immunol.*, 4, 794–800.
- Weis, W. I., Taylor, M. E., & Drickamer, K. (1998). The C-type lectin superfamily in the immune system. *Immunol. Rev.*, 163, 19–34.
- Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H., et al. (2000). A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA*, 97, 13772–13777.
- Whisstock, J. C., Silverman, G. A., Bird, P. I., Bottomley, S. P., Kaiserman, D., et al. (2010). Serpins flex their muscle: II. Structural insights into target peptidase recognition, polymerization, and transport functions. *J. Biol. Chem.*, 285, 24307–24312.
- Winans, K. A., King, D. S., Rao, V. R., & Bertozzi, C. R. (1999). A chemically synthesized version of the insect antibacterial glycopeptide, dipterucin, disrupts bacterial membrane integrity. *Biochemistry*, 38, 11700–11710.
- Yano, T., Mita, S., Ohmori, H., Oshima, Y., Fujimoto, Y., et al. (2008). Autophagic control of listeria through intracellular innate immune recognition in *Drosophila*. *Nat. Immunol.*, 9, 908–916.
- Yoshida, H., Ochiai, M., & Ashida, M. (1986). Beta-1,3-glucan receptor and peptidoglycan receptor are present as separate entities within insect prophenoloxidase activating system. *Biochem. Biophys. Res. Commun.*, 141, 1177–1184.
- Yoshida, H., Kinoshita, K., & Ashida, M. (1996). Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. *J. Biol. Chem.*, 271, 13854–13860.
- Yu, X. Q., & Kanost, M. R. (2002). Binding of hemolin to bacterial lipopolysaccharide and lipoteichoic acid. An immunoglobulin superfamily member from insects as a pattern-recognition receptor. *Eur. J. Biochem.*, 269, 1827–1834.
- Yu, X. Q., Gan, H., & Kanost, M. R. (1999). Immulectin, an inducible C-type lectin from an insect, *Manduca sexta*,

- stimulates activation of plasma prophenol oxidase. *Insect Biochem. Mol. Biol.*, 29, 585–597.
- Yu, X. Q., Jiang, H., Wang, Y., & Kanost, M. R. (2003). Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.*, 33, 197–208.
- Yu, Y., Park, J. W., Kwon, H. M., Hwang, H. O., Jang, I. H., et al. (2010). Diversity of innate immune recognition mechanism for bacterial polymeric meso-diaminopimelic acid-type peptidoglycan in insects. *J. Biol. Chem.*, 285, 32937–32845.
- Zaidman-Rémy, A., Hervé, M., Poidevin, M., Pili-Floury, S., Kim, M. S., et al. (2006). The *Drosophila* amidase PGRPLB modulates the immune response to bacterial infection. *Immunity*, 24, 463–473.
- Zamore, P. D. (2007). RNA silencing: Genomic defence with a slice of pi. *Nature*, 446, 864–865.
- Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature*, 415, 389–395.
- Zhao, M., Söderhäll, I., Park, J. W., Ma, Y. G., Osaki, T., et al. (2005). A novel 43-kDa protein as a negative regulatory component of phenoloxidase-induced melanin synthesis. *J. Biol. Chem.*, 280, 24744–24751.
- Zou, Z., Evans, J. D., Lu, Z., Zhao, P., Williams, M., et al. (2007). Comparative genomic analysis of the *Tribolium* immune system. *Genome Biol.*, 8, R177.